Novel Antibacterial Class

Peter J. Dandliker,* Steve D. Pratt, Angela M. Nilius, Candace Black-Schaefer, Xiaoan Ruan,
Danli L. Towne, Richard F. Clark, Erika E. Englund, Rolf Wagner, Moshe Weitzberg,
Linda E. Chovan, Robert K. Hickman, Melissa M. Daly, Stephan Kakavas,
Ping Zhong, Zhensheng Cao, Caroline A. David, Xiaoling Xuei,
Claude G. Lerner, Niru B. Soni, Mai Bui, Linus L. Shen,
Yingna Cai, Philip J. Merta, Anne Y. C. Saiki,
and Bruce A. Beutel

Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, Illinois 60064

We report the discovery and characterization of a novel ribosome inhibitor (NRI) class that exhibits selective and broad-spectrum antibacterial activity. Compounds in this class inhibit growth of many gram-positive and gram-negative bacteria, including the common respiratory pathogens *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Moraxella catarrhalis*, and are nontoxic to human cell lines. The first NRI was discovered in a high-throughput screen designed to identify inhibitors of cell-free translation in extracts from *S. pneumoniae*. The chemical structure of the NRI class is related to antibacterial quinolones, but, interestingly, the differences in structure are sufficient to completely alter the biochemical and intracellular mechanisms of action. Expression array studies and analysis of NRI-resistant mutants confirm this difference in intracellular mechanism and provide evidence that the NRIs inhibit bacterial protein synthesis by inhibiting ribosomes. Furthermore, compounds in the NRI series appear to inhibit bacterial ribosomes by a new mechanism, because NRI-resistant strains are not cross-resistant to other ribosome inhibitors, such as macrolides, chloramphenicol, tetracycline, aminoglycosides, or oxazolidinones. The NRIs are a promising new antibacterial class with activity against all major drug-resistant respiratory pathogens.

Respiratory tract infections are the number 1 killer worldwide, responsible for over 50 million deaths each year. Although antibacterial therapy has successfully stemmed the tide against infection since the middle of the last century, antibacterial resistance of *Streptococcus pneumoniae*, the most commonly identified pathogen associated with community-acquired pneumonia, is on the rise (2, 4, 8, 22). A recent worldwide study documents that a significant fraction of *S. pneumoniae* isolates have reduced susceptibility to penicillin (36%) and macrolides (31%) (5). Although overall rates of resistance to fluoroquinolones are low, these rates were found to be increasing rapidly in Canada (1). In general, pathogenic bacteria continuously evolve mechanisms of resistance to currently used antibacterial agents. The discovery of novel antibacterial classes would be the most powerful way to generate new therapy against these resistant pathogens. Unfortunately, novel antibacterial classes have been difficult to discover, with the oxazolidinones, identified in 1980, being the last example to successfully reach the clinic.

The bacterial ribosome is a proven target for antibacterial chemotherapy (6, 17, 20, 21). Since the 1940s, small-molecule ribosome inhibitors such as chloramphenicol, tetracyclines, macrolides, aminoglycosides, and, more recently, oxazolidinones have been used to combat bacterial infections in humans (11). These diverse chemical classes of ribosome inhibitors each bind to a different site on the ribosome, which is not surprising given its large size and complexity. Accordingly, drug resistance to each class generally develops separately, such that resistance to one class does not confer resistance to another. Therefore, from a drug discovery standpoint, the ribosome is actually a large collection of validated, broad-spectrum targets (9). A novel class of ribosome inhibitor binding to yet another ribosomal binding site should not be affected by any existing resistance mechanisms.

We attempted to discover a novel class of ribosome inhibitors by directly screening a library of compounds for this activity. This screen resulted in the identification of a novel class of antibacterial agents (novel ribosome inhibitors [NRIs]) that is unaffected by existing drug resistance mechanisms.

MATERIALS AND METHODS

Reagents. RNase-free water was used throughout. Bacterial culture media and yeast extract were purchased from Difco Laboratories, Sparks, Md. Chloramphenicol, tetracycline, minocycline, puromycin, streptomycin, cycloheximide, erythromycin, lincomycin, thiostrepton, phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), coenzyme A, dimethyl sulfoxide (DMSO), PerfectHyp hybridization solution, and spermidine were purchased from Sigma, St. Louis, Mo. Recombinant RNaseOUT RNase inhibitor, RPMI 1640 culture medium, fetal calf serum, L-glutamine, and penicillin-streptomycin (Pen/Strep) were purchased from Gibco BRL, Rockville, Md. *Hin*dIII restriction enzyme, RNase-free DNase I, SOC medium, and Superscript II reverse transcriptase were purchased from Invitrogen Corp., Carlsbad, Calif. SUPERase-In RNase inhibitor was purchased from Ambion, Inc., Austin, Tex. Slide-A-Lyzer dialysis cassettes were purchased from Pierce Biotechnology, Rockford, Ill. Plasmid pSP-luc+, S30 PreMix, complete amino acid mixture, beetle luciferin, luciferase control RNA, the RiboMAX SP6 in vitro transcription system (used for all in vitro transcription reactions), and the nuclease-treated rabbit reticulocyte lysate system were purchased from Promega Corp., Madison,

* Corresponding author. Mailing address: Abbott Laboratories, Department R4CP, Building AP52-N, 200 Abbott Park Rd., Abbott Park, IL 60064-6217. Phone: (847) 938-4841. Fax: (847) 938-3403. E-mail: peter.dandliker@abbott.com.
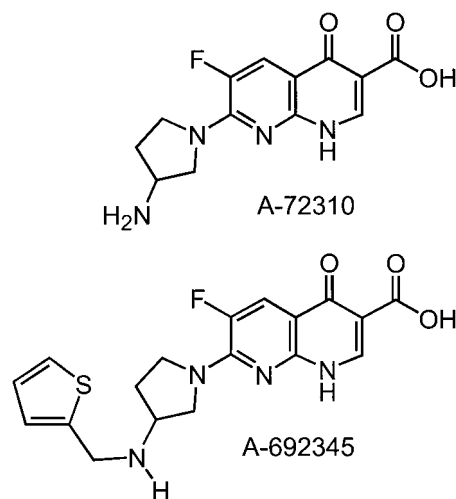
FIG. 1. Structure of NRI compounds A-72310 and A-692345.

Wis. The EcoPro T7 *Escherichia coli* extract system was purchased from Novagen, Madison, Wis. *E. coli* gyrase was purchased from TopoGEN, Inc., Columbus, Ohio. Supercoiled ColE1 DNA was isolated by a published procedure (19). Costar microtiter plates were purchased from Corning, Inc., Acton, Mass. Millipore MultiScreen-FB filter plates and Montage PCR_{96} plates were purchased from Millipore Corp., Billerica, Mass. Laemmli sample buffer was purchased from Bio-Rad, Hercules, Calif. [^{35}S]methionine, [^{3}H]UTP, [α-^{33}P]dCTP, and Sephadex G-25 spin columns were purchased from Amersham Pharmacia, Piscataway, N.J. Supermix liquid scintillation cocktail for microplates was purchased from Perkin-Elmer Life Sciences Inc., Boston, Mass. RNeasy columns and Plasmid Midi kits were purchased from Qiagen, Inc., Valencia, Calif. TaKaRa Ex *Taq* polymerase was purchased from Fisher Scientific, Pittsburgh, Pa. *Bacillus subtilis* cDNA labeling primers and Panorama gene arrays containing sequences from *B. subtilis* strain 168 were purchased from Sigma-Genosys, The Woodlands, Tex. Alamar blue reagent solution was purchased from BioSource International, Camarillo, Calif. Bacterial strains and the Jijoye human lymphoma B-cell line were obtained from the Abbott Laboratories collection or from the American Type Culture Collection, Manassas, Va. Rifampin was purchased from Calbiochem, San Diego, Calif. Oxytetracycline was purchased from Fluka, Buchs, Switzerland. Neamine was purchased from ICN Pharmaceuticals, Costa Mesa, Calif. Linezolid, ciprofloxacin, levofloxacin, norfloxacin, A-72310, and A-692345 were synthesized at Abbott Laboratories. The structures of A-72310 and A-692345 are shown in Fig. 1.

Construction of luciferase reporter plasmid pAS10rbs3. The *pA* promoter region from pEVP3 (3) was subcloned as a *Kpn*I-*Bgl*II fragment into pSP-luc+ to generate the plasmid pAS10. A new Shine-Dalgarno site (SD) composed of a consensus sequence from *S. pneumoniae* genes (10) was added with optimized spacing between the SD and the methionine start codon to yield the plasmid pAS10rbs3.

Preparation of *S. pneumoniae* S30 extract. The procedure used to prepare *S. pneumoniae* S30 extract was adapted from those described by Mackie et al. (13) and LeGault et al. (12). Five 1-liter medium bottles containing water (800 ml), Todd-Hewitt broth (30,000 mg/liter), and yeast extract (5,000 mg/liter) were each inoculated with 48 ml of *S. pneumoniae* ATCC 49619 (overnight cultures) and grown without shaking at 37°C to an optical density at 600 nm of 0.4 to 0.5. Cultures were chilled in an ethanol-ice bath for 30 min, and cells were pelleted at 6,000 × *g*. Cell pellets were pooled and washed twice with 500 ml of cold high-salt buffer (10 mM Tris HCl [pH 7.5], 1 M NH_4Cl, 15 mM Mg acetate, 50 mM KCl, 6 mM 2-mercaptoethanol) and once with 500 ml of low-salt buffer (same as high-salt buffer, except for 60 mM NH_4Cl). Cell pellets were stored at −80°C. Frozen cells were thawed for 30 min on ice water and suspended in 50 ml of low-salt buffer. Phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate were added to final concentrations of 1 and 0.1 mM, respectively, along with 1,500 U of RNaseOUT RNase inhibitor. The cells were lysed in a French press at 12,500 lb/in^2, followed by centrifugation at 4°C for 10 min at 14,000 × *g*. The supernatant was centrifuged at 4°C for 40 min at 30,000 × *g*, and the resulting extract was dialyzed in Slide-A-Lyzer cassettes (10,000 molecular weight cutoff) at 4°C against three changes (1 liter each) of low-salt buffer (twice

for 1 h each and then once overnight). The resulting extract was divided into aliquots, frozen in a dry ice-ethanol bath, and stored at −80°C. Each batch of extract was tested against a panel of different transcription and translation inhibitors and several negative controls to ensure batch-to-batch consistency.

S. pneumoniae **transcription/translation assay (luciferase readout).** Experiments were carried out in 96-well half-area black plates (Costar no. 3694) in a final volume of 16.5 μl. Test compounds were dispensed into plates as DMSO solutions and dried in a vacuum centrifuge at 42°C for 15 min. Diluted *S. pneumoniae* S30 extract (8.5 μl), composed of 2.33 μl of S30 extract, 1.17 μl of buffer A [10 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 15 mM $Mg(AcO)_2$, 50 mM KCl, 6 mM 2-mercaptoethanol], and 5 μl of H_2O, was added to each well. Plates were incubated with shaking at 25°C for 10 min, followed by addition of 7.5 μl of plasmid solution containing 5 μl of PreMix, pAS10rbs3 (0.5 μl of a 1-μg/μl solution in water), 1.25 μl of 1 mM complete amino acid mix, and 1.25 μl of H_2O. Plates were incubated at 25°C with shaking for 2 h. Reactions were stopped by adding 20 μl of kanamycin solution (20 μg of kanamycin per ml in water) to each well. Plates were analyzed on a Wallac Victor^2 plate reader by automatically injecting 50 μl of luciferin reagent (20 mM Tricine, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol [DTT], 270 μM coenzyme A, 470 μM beetle luciferin, 530 μM ATP, 1.07 mM $MgCO_3$, adjusted to pH 7.8 with NaOH) one well at a time and immediately reading luminescence after each addition.

S. pneumoniae **transcription/translation assay (electrophoretic readout).** The electrophoretic assay was identical to the coupled transcription/translation luciferase assay, with the following exceptions. Reactions involving mixtures containing 14 μl of S30, 7 μl of buffer A, 8.6 μl of H_2O, 3.5 μl of pAS10rbs3 (1 μg/μl in water), 20 μl of PreMix, 5 μl of amino acid mix (no methionine), and 24 μCi of [^{35}S]methionine (1,000 Ci/mmol) were carried out in duplicate in 1.5-ml polypropylene tubes. The contents of duplicate tubes were pooled and split: half of the volume was analyzed for luminescence as in the standard luciferase assay, and the other half was treated with 260 μl of cold acetone, incubated on ice for 15 min, and centrifuged at 21,000 × *g* for 5 min. The supernatant was aspirated, and the pellets containing precipitated protein were dried in a vacuum centrifuge. Pellets were dissolved in 80 μl of Laemmli sample buffer and electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [10% polyacrylamide]) gel. Gel visualization and band quantitation were performed on a Molecular Dynamics Storm 860 PhosphorImager with the supplied software.

S. pneumoniae **translation assay (luciferase readout).** The mRNA-driven translation assay was identical to the coupled transcription/translation luciferase assay, with the following exceptions. mRNA encoding the luciferase gene was synthesized from linearized (*Eco*RI or *Eco*RV) pAS10rbs3 plasmid and used instead of the pAS10rbs3 plasmid. Each batch of mRNA was assayed for its ability to generate luminescence signal in the assay, and the assay concentration was adjusted accordingly prior to routine use; about 500 ng of mRNA per well was typical.

E. coli **translation assay (β-galactosidase readout).** Reagents and methods from the Novagen EcoPro T7 extract system were used with modifications. mRNA was synthesized from a linearized (*Hin*dIII) plasmid containing a β-galactosidase gene. Reactions were performed in 96-well, clear, flat-bottom plastic plates (Costar no. 3596). EcoPro T7 extract (14 μl) was added to each well, and plates were agitated at room temperature on a platform shaker. After 10 min, 16 μl of mRNA solution containing β-galactosidase mRNA (2.5 μg in water) and SUPERase-In (0.7 μl, 14 U) was added to each well. The plates were covered with 96-well plate lids (Costar no. 3080) and agitated at room temperature on a platform shaker. After 30 min, 20 μl of kanamycin solution and 50 μl of β-galactosidase substrate buffer (200 mM sodium phosphate [pH 7.3] containing 2 mM $MgCl_2$, 100 mM 2-mercaptoethanol, and 4.41 mM ONPG) were added, and the plates were incubated at 37°C. After 20 min, 150 μl of 1 M Na_2CO_3 was added to stop β-galactosidase activity, and plates were read for A_{420} on a Wallac Victor^2 plate reader. Control wells containing no mRNA generated a background signal that was subtracted from test well values prior to calculation of the 50% inhibitory concentration (IC_{50}).

Eukaryotic translation assay (luciferase readout). The Promega rabbit reticulocyte lysate system was used as directed with minor modifications. Experiments were carried out in 96-well, half-area, black plates (Costar no. 3694) in a final volume of 16.5 μl. Test compounds were dispensed into plates as DMSO solutions and dried in a vacuum centrifuge at 42°C for 15 min. Rabbit reticulocyte lysate (11.5 μl) was added to each well, and plates were incubated at room temperature. After 10 min, 5 μl of a solution containing luciferase control RNA (0.033 μg; 0.033 μl of a 1-mg/ml solution in water), RNaseOUT (13.2 U; 0.33 μl of a 40-U/μl solution), complete amino acid mixture (0.33 μl), and water (4.3 μl) was added to each well, and the plates were incubated at 25°C for 2 h with shaking. Reactions were stopped by adding 20 μl of cycloheximide solution (100 μg/ml in water), and the plates were read as described for the *S. pneumoniae* transcription/translation assay.

TABLE 1. In vitro data for NRI compounds and control antibacterials

Antibacterial compound	IC$_{50}$ (μM)				MIC (μg/ml)		Jijoye B-cell cytotoxicity LD$_{50}$ (μg/ml)[c]
	S. pneumoniae transcription/ translation	*S. pneumoniae* translation	*E. coli* translation	Eukaryotic translation	*S. pneumoniae* ATCC 6303[a]	*S. pneumoniae* ATCC 7257[b]	
A-72310	10	5	5	>100	>64	32	>100
A-692345		14	19	>100	32	8	>100
Chloramphenicol	10	3.4	0.5	>100	2	1	>100
Linezolid	1.6	2.9	1.2	>100	2	0.5	>100
Minocycline		1.2	3.5		>0.06	>0.06	20
Oxytetracycline		5.4	30		0.5	0.125	>100
Tetracycline	0.35	0.55	2.6	>100	0.5	0.125	50
Erythromycin	0.3	0.4	0.25	>100	≤0.06	≤0.06	>100
Lincomycin		0.2	10.9		0.125	≤0.06	>100
Neamine		0.65	4		32	16	>100
Puromycin		0.1	0.2		1	1	<0.8
Streptomycin	<0.02	0.03	0.04	>100	>64	16	>100
Thiostrepton		0.2	0.5		≤0.06	≤0.06	>50
Rifampicin	0.1	>100	>100		≤0.06	≤0.06	>100
Cycloheximide	>100	>100	>100	0.3	>64	>64	1.5
Ciprofloxacin	>100	>100	>100	>100	0.5	8	70
Levofloxacin		>100	>100		1	16	>100
Norfloxacin		>100	>100		1	32	110

[a] Quinolone susceptible.
[b] Quinolone resistant.
[c] LD$_{50}$, 50% lethal dose.

E. coli gyrase DNA cleavage assay. For the *E. coli* gyrase DNA cleavage assay, reaction mixtures contained 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl$_2$, 2 mM DTT, 1 mM ATP, 1.8 mM spermidine, 100 μg of bovine serum albumin per ml, 0.1 μg of supercoiled ColE1 DNA, 3.3 U of gyrase enzyme, 6.5% (wt/vol) glycerol, and various concentrations of the test compound. Otherwise, conditions were identical to those described by Saiki et al. (18).

Jijoye B-cell toxicity assay. Log-phase Jijoye cells were suspended in fresh culture medium (RPMI 1640 containing 10% fetal calf serum, 1% L-glutamine, and 1% Pen/Strep) at a density of 10^6 cells/ml and deposited in 96-well plates (Costar no. 3596) at 200 μl per well. Compounds were dissolved in DMSO, serially diluted, and added to the cells over a final concentration range from 0.8 to 100 μg/ml, while maintaining a final DMSO concentration of 1%. The plates were incubated at 37°C in an atmosphere containing 5% CO$_2$ for approximately 24 h, and the effect on cellular respiration was evaluated by using Alamar blue (16). Alamar blue reagent solution, prewarmed to 37°C, was added (10 μl/well), and incubation was continued for an additional 4 h. Plates were fluorometrically assayed (λ_{ex} = 530 nm, λ_{em} = 572 nm) on a Wallac Victor^2 plate reader. Vehicle controls (untreated cells containing 1% DMSO) and positive control cytotoxins were run in each experiment.

S. pneumoniae transcription assay. Compounds were tested in V-bottom polypropylene 96-well plates (Costar no. 3363). Test compounds were dispensed into plates as DMSO solutions and dried in a vacuum centrifuge at 42°C for 15 min. *S. pneumoniae* S30 extract (2.3 μl per well) was dispensed, and the plates were incubated at room temperature for 10 min. Plasmid solution (7.5 μl) comprising 1 μg of pAS10rbs3 (1 μl of a 1-μg/μl solution in water), 3.5 μl of PreMix, 1 μl of [^{3}H]UTP (1 mCi/ml), and 2 μl of water was added to each well, and plates were covered with plastic wrap and incubated at room temperature. After 1 h, the contents of the wells were transferred to the corresponding wells of a MultiScreen-FB filter plate, each containing 200 μl of 5% trichloroacetic acid (TCA), to precipitate total protein. Plates were refrigerated for at least 1 h and then vacuumed dry on a plate manifold. Wells were washed with 5% TCA (three times at 200 μl each) and 95% ethanol (once at 200 μl). Excess ethanol was removed by blotting, and plates were allowed to air dry. One hundred microliters of liquid scintillant was added to each well, and the plates were covered, agitated, and read on a Wallac Trilux 1450 Microbeta scintillation counter.

Antibacterial susceptibility determination. Organisms were tested by the broth microdilution method as described by the National Committee for Clinical Laboratory Standards (14).

B. subtilis gene expression array experiments. Ten milliliters of Luria-Bertani (LB) broth was inoculated with *B. subtilis* 2521 (frozen glycerol stock) and incubated at 37°C overnight. The cells were diluted 1:100 (vol/vol) in fresh LB medium and grown at 37°C to an optical density of 0.6 at 600 nm. The cell

suspension was delivered in 2-ml aliquots to sterile polypropylene tubes containing DMSO solutions of individual test compounds. In all cases, including the vehicle control, the final DMSO concentration was 1.25%. After 90 min, the cells were harvested by centrifugation at 4°C for 2 min at 18,000 × *g*, and total RNA was immediately isolated with RNeasy columns, as directed by the manufacturer. The resulting RNA was treated with RNase-free DNase I for 15 min to completely eliminate chromosomal DNA, purified a second time with RNeasy columns, and quantitated spectrophotometrically. Radioactive cDNA probes were prepared by using the purified total RNA as a template. Reverse transcription reaction mixtures contained 1 μg of total RNA, [α-^{33}P]dCTP (5 μl of a 10-μCi/μl stock), 200 U of Superscript II reverse transcriptase, 4 μl of *B. subtilis* cDNA labeling primers consisting of a mixture of open reading frame-specific oligonucleotides, and sufficient Superscript II buffer to bring the final volume to 60 μl. Reaction tubes were placed in a Perkin-Elmer GeneAmp (PCR System 9600) thermal cycler that had been preheated to 90°C. After 4 min, the temperature was ramped linearly to 42°C over 20 min and then held constant at 42°C for an additional 2 h. The radiolabeled cDNAs were separated from unincorporated nucleotides by using Sephadex G-25 spin columns. Prior to hybridization, *B. subtilis* Panorama gene array membranes were washed with 1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH$_2$PO$_4$, and 1 mM EDTA [pH 7.7]) (twice at 350 ml each), blotted on filter paper to remove excess liquid, and placed individually into polyester bags (8 by 12 in.) containing 20 ml of PerfectHyp hybridization solution. Bags were immersed in a water bath at 64°C with constant shaking (120 rpm). After 2 to 3 h, the bags were drained, and a solution of radiolabeled cDNA (approximately 30 to 40 μCi) in 20 ml of fresh hybridization solution was added to each bag. Hybridization to the membrane was carried out for 2 to 3 days in the 64°C bath. The arrays were washed at room temperature with 0.1× SSPE containing 0.5% SDS (twice for 10 min each) and at 64°C (twice for 10 min each), blotted on filter paper to remove excess liquid, covered with plastic wrap, and exposed to phosphorimaging screens for 2 to 3 days. The screens were scanned at a 50-μm resolution on a Molecular Dynamics Storm 860 PhosphorImager with the supplied software. To graphically process the data, the inverse image of the array derived from drug-treated cells (light spots where radiolabeled mRNAs had hybridized) was made semitransparent (50% opacity in Adobe Photoshop) and overlaid on the array derived from untreated cells (dark spots where radiolabeled mRNAs had hybridized).

Spot intensities were also quantified in ImageQuant 5.0 (Molecular Dynamics [now Amersham Biosciences, Piscataway, N.J.]) Spots in the arrays corresponding to mRNAs of interest were manually selected, and the average pixel volume in each spot (raw value) was determined. Spot areas used for the quantitation of each mRNA were equal in both the inhibitor-treated and vehicle-treated controls. The average pixel intensity of a blank region in each array was subtracted from raw values for all of the spots in that image to yield background-corrected

TABLE 2. Antibacterial spectrum of NRI compounds

NRI	MIC (μg/ml)							
	S. pneumoniae 6303	S. pneumoniae 7257	H. influenzae GYR 1435	S. aureus 6538P	M. catarrhalis 2604	E. faecalis PIU 1967	E. coli 5358	P. aeruginosa 5007
A-72310	>64	32	4	>64	4	>64	>64	>64
A-692345	16	4	1	16	0.5	16	>64	>64
Levofloxacin	1	16	0.015	0.12	≤0.12	1	ND[a]	0.5

[a] ND, not determined.

values. Values were then normalized to account for differences in total intensity between control and drug-treated arrays. The intensity value for each spot in the drug-treated array image divided by the intensity value for the corresponding spot in the untreated array image gave the fold change values listed in Table 4.

RESULTS AND DISCUSSION

Discovery and characterization of biochemical properties of A-72310. In an effort to identify novel inhibitors of bacterial translation, we executed a cell-free transcription/translation high-throughput screen using extract from the gram-positive pathogen S. pneumoniae. Although these experiments would have been easier and less expensive to conduct with extracts from E. coli, we opted for the relevant system to maximize the probability of identifying a lead series with specific utility against this important pathogen.

S. pneumoniae S30 extract was used to catalyze coupled transcription/translation of a plasmid-borne luciferase gene, encoding an mRNA with optimized ribosome binding site and

TABLE 3. Antibacterial activity of A-692345 against clinically resistant S. pneumoniae and S. aureus

Organism	Resistance[a]	MIC (μg/ml)	
		A-692345	Levofloxacin
S. pneumoniae			
ATCC 6303		32	0.5
ATCC 49619	PEN	16	1
ATCC 5957	PEN	16	1
ATCC 7257	LVX	4	8
ATCC 6396	ERY[b], CLX, TET	16	1
ATCC 5649	PEN, ERY[c]	8	4
S. aureus			
ATCC 29213		32	0.25
ATCC 1350	PEN, ERY, CLX	32	0.12
ATCC 7667	PEN, OXA	32	0.25
ATCC 7702	PEN, OXA, CHL, TET, GEN, CIP	32	16
ATCC 2175	PEN, OXA, ERY, CLX, TET, GEN, CHL	32	0.25
ATCC 7681	PEN, OXA, ERY, CLX, CHL, GEN, CIP	32	8
ATCC 3405	PEN, OXA, ERY, CLX, TET, GEN, CIP	32	16

[a] Resistance phenotype determined by NCCLS criteria (data not shown). Tested antibiotics included penicillin (PEN), erythromycin (ERY), clindamycin (CLX), tetracycline (TET), chloramphenicol (CHL), and levofloxacin (LVX) for *S. pneumoniae* and penicillin, oxacillin (OXA), erythromycin, clindamycin, chloramphenicol, tetracycline, gentamicin (GEN), and ciprofloxacin (CIP) for *S. aureus*.
[b] *erm* (B)
[c] *mef* (A)

spacing. Activity of the resulting luciferase protein was quantified in a luminescence assay. As expected, compounds inhibiting transcription, translation, or the luminescence reaction itself reduced the luminescence output relative to the uninhibited control. A battery of control compounds, including the transcription inhibitor, rifampin, and translation inhibitors, chloramphenicol, linezolid, tetracycline, erythromycin, and streptomycin, was used to validate the assay (Table 1). The goal of the screen and subsequent characterization of hits was to identify compounds capable of inhibiting bacterial, but not eukaryotic RNA or protein synthesis. Ideally, these would therefore also exhibit selective antibacterial cell activity. Out of a screening library of ~300,000 small molecules, only one compound, A-72310, had this desirable profile of selectively inhibiting the bacterial coupled assay (IC$_{50}$ = 10 μM).

To dissect the mechanism of action of A-72310, we used an uncoupled cell-free S. pneumoniae translation assay, initiated by the addition of mRNA encoding luciferase. In contrast to the coupled transcription/translation system, this assay is sensitive only to compounds that inhibit translation and is not affected by those that block transcription. A-72310 showed potent inhibitory activity (IC$_{50}$ = 5 μM), as did all of the positive control ribosome inhibitors (Table 1). This inhibitory activity is within the experimental error of that observed in the coupled assay and is sufficient to account for the activity observed in the coupled assay.

To prove A-72310 did not also inhibit RNA synthesis, we tested it in a cell-free S. pneumoniae transcription assay, monitoring incorporation of radiolabeled uridine into RNA. In contrast to the transcription inhibitors rifampin and streptolydigin (IC$_{50}$ = 0.1 and 1 μM, respectively), control translation inhibitors and A-72310 were inactive. (Table 1)

We conducted experiments to eliminate the possibility that A-72310 disrupts the luminescent assay readout by directly inhibiting the luciferase enzymatic activity or quenching the luminescence reaction. In particular, we ran uninhibited translation reactions to generate luciferase in situ, stopped translation with kanamycin, and then added A-72310. A-72310 did not inhibit light output in these tests, showing that it does not inhibit luciferase enzymatic activity or quench luminescence. Furthermore, when aliquots of coupled transcription/translation reaction mixtures containing A-72310 and [^{35}S]methionine were electrophoresed, the inhibition of luciferase production measured on the gel was the same as that observed when using the luminescence output (data not shown).

Because of our interest in broad-spectrum agents, we tested A-72310 in a bacterial translation assay using cell extracts from E. coli. For this purpose, we used a commercially available E. coli extract system from Novagen charged with mRNA encod-
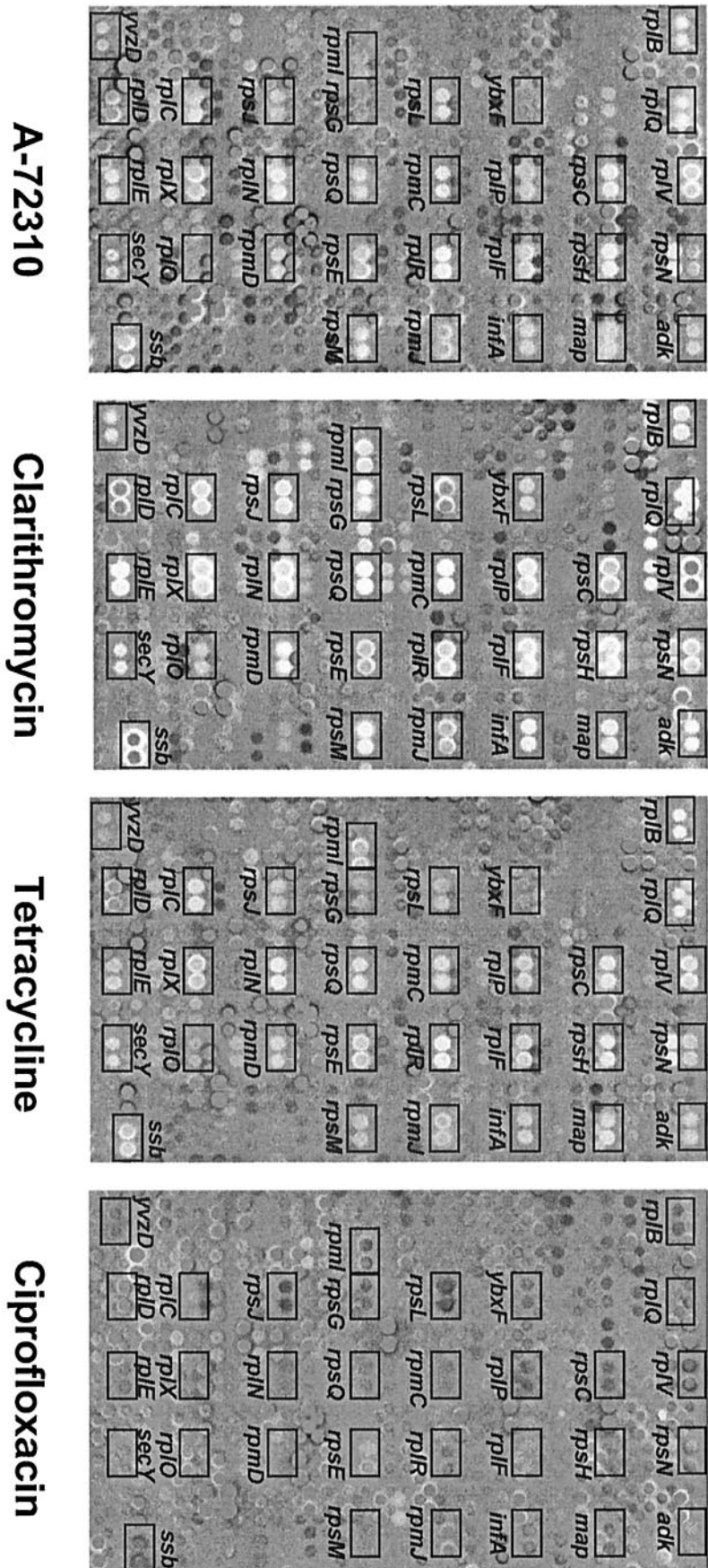
FIG. 2. Composite *B. subtilis* gene expression array images for A-72310 (128 μg/ml), clarithromycin (10 μg/ml), tetracycline (0.1 μg/ml), and ciprofloxacin (0.1 μg/ml). Drug-induced changes in mRNA concentration are indicated by either light (upregulated), dark (downregulated), or neutral gray (unchanged) spots (in duplicate).

TABLE 4. Quantitative analysis of expression array data for A-72310-, tetracycline-, clarithromycin-, and ciprofloxacin-treated *B. subtilis*

Functional category and gene name	Protein	Fold change[a]			
		A-72310	Tetracycline	Clarithromycin	Ciprofloxacin
Ribosomal proteins					
rplB	Ribosomal protein L2 (BL2)	34.3	38.6	14.8	0.6
rplC	Ribosomal protein L3 (BL3)	6.4	7.1	11.8	0.5
rplD	Ribosomal protein L4	2.9	2.0	4.7	1.0
rplE	Ribosomal protein L5 (BL6)	13.6	5.8	17.4	1.2
rplF	Ribosomal protein L6 (BL8)	7.7	7.1	10.8	1.4
rplJ	Ribosomal protein L10 (BL5)	1.8	1.4	6.1	1.1
rplK	Ribosomal protein L11 (BL11)	4.3	2.2	6.1	0.5
rplL	Ribosomal protein L12 (BL9)	7.9	6.7	16.2	0.7
rplN	Ribosomal protein L14	24.8	16.8	17.2	0.6
rplO	Ribosomal protein L15	1.8	1.6	4.1	1.0
rplP	Ribosomal protein L16	5.6	4.9	18.3	0.5
rplQ	Ribosomal protein L17 (BL15)	36.6	27.5	10.4	0.9
rplR	Ribosomal protein L18	46.5	42.1	19.0	0.8
rplT	Ribosomal protein L20	10.9	25.3	11.8	0.6
rplV	Ribosomal protein L22 (BL17)	33.6	9.8	21.8	0.4
rplW	Ribosomal protein L23	37.4	32.9	12.7	0.4
rplX	Ribosomal protein L24 (BL23) (histone-like protein HPB12)	10.1	7.7	15.5	0.8
rpmA	Ribosomal protein L27 (BL24)	4.0	4.1	4.1	0.9
rpmC	Ribosomal protein L29	23.8	16.6	22.4	0.6
rpmD	Ribosomal protein L30 (BL27)	9.4	8.3	16.7	0.7
rpmI	Ribosomal protein L35	2.5	4.6	8.4	0.7
rpmJ	Ribosomal protein L36 (ribosomal protein B)	6.0	4.5	4.5	1.0
rpsC	Ribosomal protein S3 (BS3)	24.5	11.0	22.1	0.7
rpsD	Ribosomal protein S4 (BS4)	0.6	1.1	4.4	0.4
rpsE	Ribosomal protein S5	7.7	9.1	7.2	1.8
rpsG	Ribosomal protein S7 (BS7)	1.7	1.8	8.1	0.6
rpsH	Ribosomal protein S8 (BS8)	21.0	10.5	19.7	0.9
rpsJ	Ribosomal protein S10 (BS13)	8.6	7.8	8.5	0.3
rpsL	Ribosomal protein S12 (BS12)	6.9	3.0	5.8	0.4
rpsM	Ribosomal protein S13	13.6	6.5	9.6	0.8
rpsN	Ribosomal protein S14	4.1	4.6	11.0	1.0
rpsQ	Ribosomal protein S17 (BS16)	23.9	19.2	20.5	0.8
rpsS	Ribosomal protein S19 (BS19)	11.5	7.2	19.0	0.4
ybxF	Similar to ribosomal protein L7AE family	1.6	1.5	5.3	4.9
Translation factors					
efp	Elongation factor P	2.6	0.5	0.1	0.7
frr	Ribosome recycling factor	3.1	1.1	1.4	0.4
fus	Elongation factor G	3.4	1.4	9.9	1.3
infA	Initiation factor IF-1	9.4	6.0	8.0	0.9
infB	Initiation factor IF-2	3.0	1.9	1.1	1.0
infC	Initiation factor IF-3	2.3	3.5	4.2	1.0
prfA	Peptide chain release factor 1	0.9	0.9	1.0	0.8
prfB	Peptide chain release factor 2	1.8	1.0	1.2	0.8
tufA	Elongation factor Tu	9.6	3.0	1.8	1.0
ykrS	Similar to initiation factor eIF-2B (alpha subunit)	4.7	5.5	0.6	1.3
ylaG	Similar to GTP-binding elongation factor	3.2	2.1	0.4	0.2
tRNA synthetases					
glyQ	Glycyl-tRNA synthetase (alpha subunit)	1.7	1.8	1.0	0.9
glyS	Glycyl-tRNA synthetase (beta subunit)	3.5	1.3	0.2	0.7
hisS	Hlstidyl-tRNA synthetase	2.0	1.4	0.7	0.8
ileS	Isoleucyl-tRNA synthetase	3.9	0.2	0.5	0.1
pheS	Phenylalanyl-tRNA synthetase (alpha subunit)	2.8	1.1	0.6	1.1
pheT	Phenylalanyl-tRNA synthetase (beta subunit)	1.8	1.5	0.7	1.0
serS	Seryl-tRNA synthetase	1.2	1.1	0.8	1.2
thrS	Threonyl-tRNA synthetase	1.8	1.7	0.7	1.4
tyrS	Tyrosyl-tRNA synthetase	1.0	2.2	1.0	0.5
valS	Valyl-tRNA synthetase	0.8	0.8	0.4	1.0
Amino acid biosynthesis					
ilvN	Acetolactate synthase (acetohydroxy-acid synthase) (small subunit)	0.7	5.6	1.0	0.6

Continued on following page

TABLE 4—*Continued*

Functional category and gene name	Protein	Fold change[a]			
		A-72310	Tetracycline	Clarithromycin	Ciprofloxacin
leuD	3-Isopropylmalate dehydratase (small subunit)	1.7	8.4	5.8	1.0
metK	S-Adenosylmethionine synthetase	3.1	2.6	1.3	1.0
trpA	Tryptophan synthase (alpha subunit)	1.0	0.5	0.6	9.8
Other categories					
adk	Adenylate kinase	10.2	9.2	10.6	1.9
lexA	Transcriptional regulator	1.0	1.0	0.8	0.9
map	Methionine aminopeptidase	6.8	5.2	9.4	0.9
recA	Multifunctional SOS repair regulator	3.8	1.8	1.8	3.3
rsbW	Switch protein/serine kinase and anti-sigma factor	5.6	3.4	1.5	3.1
rsbX	Serine phosphatase (dephosphorylation of RsbS)	6.7	5.1	0.5	3.9
ruvA	Holiday junction DNA helicase	1.5	3.1	2.5	0.7
ruvB	Holiday junction DNA helicase	0.6	1.5	1.9	1.1
secY	Preprotein translocase subunit	10.4	9.2	8.4	1.1
ssb	Single-strand DNA-binding protein	17.9	9.6	15.6	0.6
uvrA	Excinuclease ABC (subunit A)	3.1	2.1	1.1	2.4
ybyB	Unknown	2.5	2.6	0.1	6.1
yqgZ	Unknown	0.8	0.9	0.5	3.9
ywzA	Unknown	3.0	1.2	1.1	3.2

[a] Fold change relative to vehicle-treated control.

ing β-galactosidase. The β-galactosidase concentration was quantified by its enzymatic action on a chromogenic substrate. A-72310 showed potent inhibitory activity ($IC_{50} = 5$ μM), as did all the positive control ribosome inhibitors (Table 1). These data all confirm that A-72310 is a bona fide inhibitor of bacterial translation.

A-72310 was inactive in a eukaryotic translation assay (IC_{50}, >100 μM). The assay was validated with cycloheximide, a potent eukaryotic translation inhibitor. The observed biochemical selectivity of A-72310 for bacterial protein synthesis was its most unique characteristic compared to all other high-throughput screening hits.

Given its close structural similarity to antibacterial quinolones that inhibit DNA gyrase and topoisomerase IV, we were surprised that A-72310 inhibited bacterial translation. Indeed, none of the control quinolones we tested inhibited translation (Table 1). Furthermore, although thousands of related quinolone analogs were tested in the course of screening, none of them were detected as assay hits. These data support the conclusion that the structural similarity to quinolones is irrelevant to the biochemical properties of A-72310.

The vast majority of antibacterial quinolones, including thousands of other quinolones in our screening library, have an aryl or alkyl substituent at the N-1 position. Before the discovery of the NRI class, A-72310 would have been considered only as a synthetic intermediate for the corresponding N-1-substituted naphthyridine. Placement of typical antibacterial quinolone substituents at the N-1 position results in a substantial loss of translation inhibitory activity. For example, esafloxacin, the N-1 ethyl derivative of A-72310, inhibits coupled *S. pneumoniae* transcription/translation too weakly ($IC_{50} = 100$ μM) to have been detected in the screen. Conversely, addition of the N-1 ethyl substituent drastically enhances the compound's ability to form cleavable DNA-gyrase complexes, a biochemical property common to marketed quinolones (19). The propensity of a compound to induce formation of cleavable complexes can be expressed as the concentration of compound
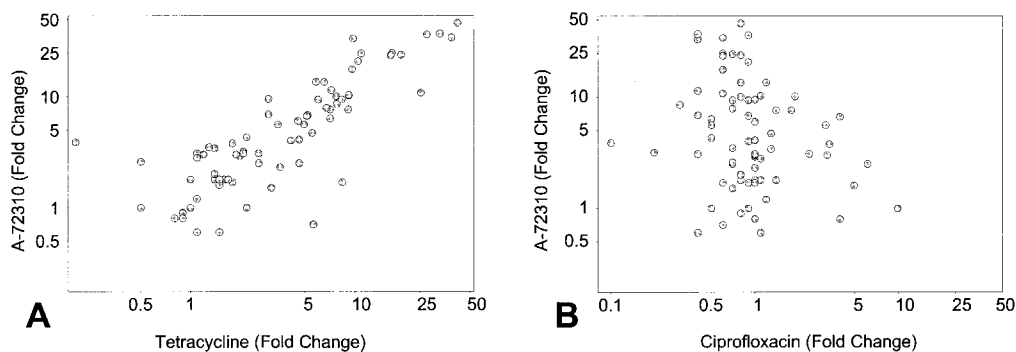

FIG. 3. Scatter plots of the fold change in *B. subtilis* mRNA levels induced by A-72310 and tetracycline (A) and A-72310 and ciprofloxacin (B).

required to induce 50% of maximal DNA linearization (CC_{50}). Whereas esafloxacin is a potent inhibitor with $CC_{50} = 0.4$ μM, A-72310 exhibits an *E. coli* gyrase $CC_{50} > 100$ μM.

A-72310 has been the subject of a medicinal chemistry effort resulting in an extensive series of ribosome inhibitors in the NRI class. Details of the structure-activity relationships (SAR) for the NRI series will be the subject of other articles, but it is worth noting that the SAR for the NRI series diverges significantly from quinolone SAR, not only at N-1, but also at many other positions.

Biological characterization of NRIs. In addition to their cell-free biochemical activity, NRIs also inhibit the growth of gram-positive and gram-negative bacteria in vitro. During the course of chemical optimization, we prepared analogs such as A-692345 that exhibit a biochemical profile similar to A-72310 and possess improved antibacterial activity (Table 1). The spectrum of antibacterial activity for A-692345, which is representative of the series, includes the key pathogens associated with respiratory tract infections (*Staphylococcus aureus*, *S. pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*), but not *E. coli* or *Pseudomonas aeruginosa* (Table 2). In addition to inhibiting susceptible strains, NRIs inhibit the growth of clinical isolates of *S. aureus* and *S. pneumoniae* with all major mechanisms of drug resistance (Table 3). MICs for antibacterial-resistant strains, including quinolone-resistant *S. pneumoniae* 7257 and *S. aureus* 3405, 7702, and 7681 (all of which have point mutations in the genes encoding DNA gyrase and topoisomerase IV) were the same as or lower than those for susceptible strains. These quinolone-resistant strains exhibit at least 16-fold reduced susceptibility to ciprofloxacin and other quinolones (data not shown). Major mechanisms of antibacterial resistance do not affect the ability of A-692435 to inhibit bacterial growth, because the NRI inhibits the ribosome via a novel mechanism.

Consistent with the lack of eukaryotic translation inhibition activity, the NRIs were inactive in a Jijoye B-cell assay (Table 1).

To prove that members of the NRI series inhibit translation in bacteria, gene expression array and mutational studies were undertaken. When administered to bacterial cells, ribosome inhibitors induce an increase in production of mRNAs encoding ribosomal protein genes (15). We used commercially available gene expression arrays to monitor global changes in mRNA levels in response to A-72310 and several control antibacterials. We made initial attempts to observe changes in gene expression by treating cells with a concentration of inhibitor equal to the MIC for *B. subtilis* 2521, measured under standard conditions. In some cases, we observed that the inhibitor concentration needed to be significantly higher in order to observe reproducible changes in gene expression. Specifically, the concentrations required to observe a reproducible pattern were as follows: clarithromycin, 10 μg/ml (MIC = 0.03 μg/ml); ciprofloxacin, 0.1 μg/ml (MIC = 0.01 μg/ml); tetracycline, 0.1 μg/ml (MIC = 0.06 μg/ml); and A-72310, 128 μg/ml (MIC = 128 μg/ml). Notably high concentrations of clarithromycin and ciprofloxacin relative to their standard MICs were required to induce reproducible gene expression changes in our experiments, while the concentration of A-72310 required to induce this effect was 128 μg/ml, equal to the MIC. At lower concentrations, A-72310 did not induce reproducible changes in gene expression, demonstrating that an especially high drug

concentration relative to the MIC is not required to induce a regulatory response in the cell.

Composite gene expression array images that graphically display the relative changes in concentration of individual *B. subtilis* mRNAs induced by antibacterial treatment are shown in Fig. 2. In the processed images, upregulated mRNAs appear as white spots, downregulated mRNAs appear as dark spots, and mRNAs whose level is unaffected by antibacterial treatment are gray and of similar intensity to the neutral gray background; each gene is arrayed in duplicate. The translation inhibitors clarithromycin, tetracycline, and A-72310 all caused the induction of ribosomal protein genes in particular, consistent with their common mechanism of action. In sharp contrast, ciprofloxacin caused no significant change in the levels of the same mRNAs. Although semiquantitative, this graphical approach is a fast and effective way to visualize patterns of mRNA regulation across the genome and to match response patterns of compounds of unknown mechanism to those of control compounds.

We also quantitatively analyzed changes in *B. subtilis* mRNA expression that occurred in response to treatment with A-72310, clarithromycin, tetracycline, and ciprofloxacin. These mRNAs encode a range of proteins, including ribosomal proteins, tRNA synthetases, translation factors, proteins involved in amino acid biosynthesis, proteins in the *pur* gene cluster, and an additional seven mRNAs from other classifications whose expression level was significantly altered (greater-than-threefold increase or decrease in mRNA level relative to the level of the vehicle-treated control) by treatment with at least one of the compounds. The relative changes in mRNA expression for all of these genes are listed in Table 4. Spots in Fig. 2 are annotated with the corresponding gene name so that spot intensity in the figure can be correlated with the fold change in Table 4. Importantly, the spots in the array can be visually rank ordered, consistent with the quantitative data. For instance, the brightest spots in Fig. 2 (A-72310) occur for *rplR* mRNA, the most highly induced message for this inhibitor (37-fold increase). Similarly, spots for *infA* and *rpsM* in Fig. 2 (A-72310) are roughly equivalent and have medium intensity (9- and 14-fold increases, respectively), while spots corresponding to *ybxF* appear unchanged, consistent with data in Table 4.

All three translation inhibitors induced changes in mRNA levels that are quantitatively different from the changes induced by ciprofloxacin. Levels of mRNA encoding 26 of the ribosomal proteins are increased by more than a factor of 3 in the A-72310-, tetracycline-, and clarithromycin-treated samples. Ten of these messengers increase by greater than an order of magnitude. These levels of upregulation are considerably higher than those observed in *S. pneumoniae* (15) but agree qualitatively. In contrast, the levels of mRNA encoding these same genes did not change significantly in the ciprofloxacin-treated cells. All of the translation inhibitors also induced mRNAs for translation initiation factor IF-1 (*infA*) and translation elongation factor G (*fus*) by more than threefold.

Contrary to the observations in *S. pneumoniae* (15), levels of mRNAs encoding genes in the *pur* cluster (*pbuX*, *purB*, *purE*, *purF*, *purH*, *purK*, *purL*, *purM*, *purN*, and *xpt*) were not significantly upregulated in these experiments (data not shown), nor did we observe any significant downregulation of any aminoacyl-tRNA synthetase mRNAs with drug treatment, although levels of a few of these mRNAs (*ileS* and *glyS*) actually in-

creased in the presence of A-72310 (Table 4). Messenger encoding most of the amino acid biosynthesis genes we analyzed was not significantly changed by any of the inhibitors. These unaffected species included *glyA*, *asd*, *dapA*, *ilvD*, *trpC*, *ilvC*, *aspB*, and *metB* (data not shown).

Messenger for genes from additional classes was also upregulated uniformly by the three translation inhibitors and not by ciprofloxacin. These included mRNA encoding a single-stranded DNA-binding protein (*ssb*), adenylate kinase (*adk*), methionine aminopeptidase (*map*), a preprotein translocase subunit (*secY*), and a gene of unknown function (*yvzd*).

In *B. subtilis*, ciprofloxacin treatment significantly induced mRNA encoding the alpha subunit of tryptophan synthase (*trpA*), an unknown protein similar to the ribosomal protein L7AE family (*ybxF*), and two unknown genes, *ybyB* and *yqgZ*. Levels of these mRNAs were not affected by any the translation inhibitors. Two messengers were also downregulated by ciprofloxacin: isoleucyl-tRNA synthetase (*ileS*) and a protein similar to GTP-binding elongation factor (*ylaG*). These were unchanged by tetracycline and clarithromycin, whereas the levels were actually increased in the case of A-72310—the exact opposite of ciprofloxacin. In *H. infuenzae*, ciprofloxacin was previously shown to induce genes encoding proteins involved in SOS repair (*recA*, *uvrA*, and *lexA*), DNA helicases (*ruvA*, *ruvB*), and the excinuclease ABC subunit A (*uvrA*) (7). Of these genes in *B. subtilis*, ciprofloxacin only caused a significant, but modest increase for *recA*, which was also apparent in the A-72310-treated samples. Taken together, these data demonstrate that genes upregulated by ciprofloxacin in *B. subtilis* are not generally upregulated by the NRI or other translation inhibitors and support the conclusion that the NRI acts by a non-quinolone mechanism in the cell.

Scatter plots of the fold change in mRNA levels induced by A-72310 and tetracycline further demonstrate that the patterns of gene expression induced by these two inhibitors very closely resemble each other (Fig. 3A). Conversely, there is no correlation in the data for A-72310 and ciprofloxacin (Fig. 3B). The results support the conclusion that A-72310 inhibits translation in *B. subtilis* and does not induce a cellular response similar to that of ciprofloxacin

To further understand the NRI mechanism, we carried out experiments to isolate and characterize NRI-resistant *S. pneumoniae* point mutants. *S. pneumoniae* strains resistant to compounds in the NRI series could be selected in the laboratory, albeit at low frequency ($\leq 1 \times 10^{-8}$). Resistance arose from genetically transferable point mutations in the 16S rRNA gene and the S3 protein. These mutants, while two- to fivefold resistant to multiple compounds in the NRI series, are not cross-resistant to chloramphenicol, linezolid, erythromycin, tetracycline, oxytetracycline, spectinomycin, streptomycin, puromycin, or lincomycin—indicating that NRI class is likely to interact with the ribosome at a new site (A. M. Nilius and R. K. Hickman, personal communication).

The NRI class is a novel antibacterial class that inhibits translation in cell extracts from gram-positive and gram-negative organisms and is biochemically selective versus eukaryotic translation. Compounds in this class inhibit translation in bacterial cells, as evidenced by expression array and mutational experiments, and, despite some chemical similarity, are mechanistically distinct from quinolones, since drug-resistant strains

for each class are not cross-resistant with each other. In summary, the NRI is a promising novel antibacterial class, and further chemical optimization could yield a new antibacterial class for treatment of respiratory and other infections.

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