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Regulated Expression of the *Escherichia coli lepB* Gene as a Tool for Cellular Testing of Antimicrobial Compounds That Inhibit Signal Peptidase I In Vitro

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***Escherichia coli* under-expressing *lepB* was utilized to test cellular inhibition of signal peptidase I (SPase). For the construction of a *lepB* regulatable strain, the *E. coli lepB* gene was cloned into pBAD, with expression dependent on L-arabinose. The chromosomal copy of *lepB* was replaced with a kanamycin resistance gene, which was subsequently removed. SPase production by the *lepB* regulatable strain in the presence of various concentrations of L-arabinose was monitored by Western blot analysis. At lower arabinose concentrations growth proceeded more slowly, possibly due to a decrease of SPase levels in the cells. A penem SPase inhibitor with little antimicrobial activity against *E. coli* when tested at 100 μM was utilized to validate the cell-based system. Under-expression of *lepB* sensitized the cells to penem, with complete growth inhibition observed at 10 to 30 μM. Growth was rescued by increasing the SPase levels. The cell-based assay was used to test cellular inhibition of SPase by compounds that inhibit the enzyme in vitro. MD1, MD2, and MD3 are SPase inhibitors with antimicrobial activity against *Staphylococcus aureus*, although they do not inhibit growth of *E. coli*. MD1 presented the best spectrum of antimicrobial activity. Both MD1 and MD2 prevented growth of *E. coli* under-expressing *lepB* in the presence of polymyxin B nonapeptide, with growth rescue observed when wild-type levels of SPase were produced. MD3 and MD4, a reactive analog of MD3, inhibited growth of *E. coli* under-expressing *lepB*. However, growth rescue in the presence of these compounds following increased *lepB* expression was observed only after prolonged incubation.**

The development of antibiotic-resistant bacteria as well as the emergence of new pathogens has created a need for novel antimicrobial drugs. Microbial genome sequencing efforts have focused on the identification of essential genes, some of which code for membrane-bound proteins of unknown function. Cell-based assays utilizing strains under-expressing target genes may provide a means for identifying inhibitors of novel proteins in the absence of known function or of an in vitro biochemical assay.

Signal peptidase I (SPase) is an essential enzyme for many microorganisms. *Escherichia coli* has only one gene (*lepB*) that codes for a catalytically active SPase (11). Repression of *lepB* expression by an arabinose (Ara) promoter (10) or by partial deletion of the natural promoter (11) results in cessation of cell growth and division. The *Staphylococcus aureus spsB* gene encodes an active SPase (8). Experiments in which the *spsB* gene was cloned into a plasmid that is temperature sensitive for replication indicated that *spsB* is also essential for growth. An open reading frame immediately upstream of the *spsB* gene encodes a homologous sequence and was predicted to be devoid of catalytic activity (8).

Many membrane and secretory proteins in both eukaryotic and prokaryotic cells are synthesized as precursors with an N-terminal signal peptide containing 15 to 30 amino acids. SPases catalyze the processing of N-terminal signal peptides, thereby allowing the release of exported proteins from membranes (9, 12). The bacterial SPases consist of single polypeptides anchored to the membrane by one or two transmembrane domains. The best-characterized SPase is from *E. coli*, which spans the membrane twice (1, 9). SPases are irreversibly inhibited by certain penem compounds, which act by acylation of the active site serine (6, 17). The crystal structure of a catalytically active soluble fragment of the *E. coli* enzyme has been described in complex with a β-lactam (5S, 6S penem) (17). The SPase structure is consistent with the use of Lys 145 as a general base in the activation of the nucleophilic active site Ser 90 (5).

SPase biochemical assays are available (7, 14, 26), but no compounds that effectively inhibit SPase both in vitro and in vivo have been described to date. An efficient synthetic substrate for SPase was recently reported, which presents a k_{cat}/K_m ratio of 2.5×10^6 M^{-1} s^{-1} (20). However, SPase inhibition in vitro by a given compound does not necessarily correlate with antimicrobial activity. The relevance of biochemical screens is further complicated by the indication that the SPase active site may be partially submerged in the lipid bilayer (23), making its active site somewhat inaccessible to compounds screened in vitro. Here we describe SPase inhibitors obtained with a biochemical assay and the development of a cell-based assay that allowed for investigation of specific cellular inhibition of the target.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* TOP10 and plasmid pBAD-HisA (13) were obtained from Invitrogen (San Diego, Calif.). *E. coli* DY329 (24) was genetically modified for the construction of a knockout strain. All other bacterial strains were from the American Type Culture Collection. Plasmid pJDP8 is a derivative of pSC101 containing the *cre* gene (21).

Cloning of the *E. coli lepB* gene. The *lepB* gene from *E. coli* ATCC 47076 (12, 15) was PCR amplified and inserted into the *Bam*HI and *Nde*I sites of plasmid pET26(+) (Novagen) as previously described (20) to generate pMB1. pMB2 was engineered by inserting *E. coli lepB* into the *Nco*I site of pBAD-His (Invitrogen) after amplification with the following primers: forward, 5′CATGCCATGGATGGCGAATATGTTTGCCCTGA 3′; reverse, 5′CATGCCATGGAGATGGTATTAATGGATGCCG 3′. Chromosomal DNA and plasmid isolation, DNA desalting, and purification from agarose gels were performed with Qiagen kits. DNA transformation into *E. coli* was performed either according to instructions from the manufacturer or by following standard techniques (18).

Western blot analysis. Wild-type *E. coli* SPase was purified as previously described (20). Polyclonal antibodies against SPase were produced in a rabbit by Research Genetics, Inc. (Huntsville, Ala.). After centrifugation, the 10-week bleed was subjected to ammonium sulfate precipitation followed by affinity purification with protein G (Boehringer Mannheim, Indianapolis, Ind.). For Western blot analysis, the proteins in the cell extracts were separated by sodium dodecyl sulfate electrophoresis in gradient gels (4 to 20% acrylamide; Invitrogen) according to instructions from the manufacturer. The samples from *E. coli* TOP10 cells over-expressing *lepB* were prepared by freezing and thawing followed by boiling with sodium dodecyl sulfate-containing buffer (18). For expression analysis of the *E. coli lepB* regulatable strain, DNase (Gibco BRL) was added to the cells, which were then lysed with a French press at 12,000 lb/in^2 and processed as described above (18). After transfer to nitrocellulose membranes, the Western blot was processed using anti-rabbit alkaline phosphatase-conjugated antibodies (18).

Construction of a *lepB* regulatable strain. The *E. coli* strain we used (strain 391) is a derivative from DY329 that had the *araCBAD* operon knocked out and was therefore unable to metabolize *ara*. The bacteriophage λ recombination system was used to promote homologous recombination (16, 24). The antibiotic markers were removed by utilizing the bacteriophage P1 site-specific recombination system *cre-loxP* (21). The primers used to amplify a kanamycin (KAN) resistance gene from a linear *loxP*-KAN cassette contained the *loxP* sites (underlined) and *lepB* flanking sequences (in italic) as follows: forward primer, 5′ *GGAAGCGTTCCTCGCCATTCTGCACGTCGGCAAAGACAACAAATAACC CTTAGGAGTTGG*TATCACGAGGCCCTTTCGTCTT 3′; reverse primer, 5′ *TAGCCACGGGAGATTTATCTCATAAATAATTCACGTTGTCGCCATAACG GCGACAACGTG*TTTTCACCGTCATCACCGAAAC 3′. Electroporation-competent (24) *E. coli* 391 cells containing pBAD with *E. coli lepB* were transformed with the linear *loxP*-KAN cassette, and recombinants were selected on Luria-Bertani (LB) agar containing 0.2% Ara, 30 μg of KAN per ml, and 50 μg of ampicillin (AMP) per ml. The resulting colonies were tested for growth on plates containing antibiotics, with and without Ara. The colonies growing exclusively on Ara-containing agar were tested by PCR, with distal primers designed according to the *lepB* flanking genes *rnc* and *lepA* (forward primer, 5′ TAATCCGGCAGAAAAGGCGCT 3′; reverse primer, 5′ TACTGCTGGCACTACGATGA 3′). The KAN resistance gene used to replace the chromosomal copy of *lepB* in a strain expressing *E. coli lepB* from pBAD was removed by transforming the cells with pJDP8 followed by selection on LB agar containing Ara, spectinomycin, and AMP. Loss of the KAN resistance mark was ascertained by streaking the cells on LB medium containing Ara, KAN, and AMP. Spectinomycin-resistant, AMP-resistant, and KAN-sensitive cultures were then transferred to LB medium containing Ara and AMP and incubated at 37°C to obtain isolated colonies, which were again tested for loss of the KAN resistance marker.

Growth studies. Growth of *E. coli* (*E. coli* parent strain containing or not containing pBAD-HisA and a *lepB* regulatable strain) was investigated in 50-ml Falcon tubes containing 3 ml of LB medium or RM minimal medium (18). Incubation was done at 32°C and 250 rpm. Growth was monitored by measuring the optical density at 600 nm, after 10 and 20 h of incubation. The effect of SPase inhibitors on *E. coli* TOP10 or the *lepB* regulatable strain containing the *E. coli lepB* gene cloned into pBAD was tested in the presence of various concentrations of polymyxin B nonapeptide (Pbn) (22). Further tests with compounds that inhibit growth of the *lepB* regulatable strain (cell-based assay) utilized 96-well plates, with 150 μl of medium added per well. Plates were incubated with agitation at 32°C and optical density was determined at intervals. Inocula were prepared by transferring the cells from frozen stocks to LB agar containing AMP and Ara. Various concentrations of Ara were tested for inoculum preparation.

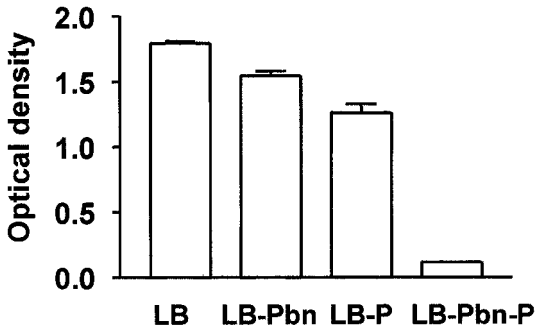

FIG. 1. Growth of *E. coli* TOP10 bearing pMB2 was investigated in LB medium (LB) and LB medium supplemented with 20 μg of Pbn per ml (LB-Pbn), 100 μM penem (LB-P), or both Pbn and penem (LB-Pbn-P). Growth was measured as the increase in optical density at 600 nm after 20 h of incubation. The initial optical density of the inoculum was 0.1. Experiments were performed in duplicate.

Drug susceptibility testing. MICs were determined for a panel of microorganisms (4). In brief, bacterial cultures were inoculated in 96-well plates containing liquid medium with various concentrations of the test compounds. Growth was monitored by measuring the optical density of the culture after incubation at 37°C for 24 h.

Synthesis of penem and MD4 and stability tests for SPase inhibitors. Penem 64 was synthesized as previously described (2, 3, 6). MD4 is a reactive analog of MD3, synthesized to test the effect of a possible breakdown product of MD3. MD4 was synthesized by the addition of freshly prepared vinylmagnesium bromide to 2,5-dichlorobenzaldehyde in tetrahydrofuran (THF) and oxidation of the resulting alcohol with MnO_2 in CH_2Cl_2. Stability tests were performed with the SPase inhibitors MD1, MD2, and MD3 dissolved in dimethyl sulfoxide (DMSO), methanol, or Tris buffer. The compounds were incubated at room temperature, and degradation was assessed after various incubation times.

SPase biochemical assay and IC_{50} determinations. SPase and K_5L_{10}YFSASALA~KIK(fluorescein)NH_2 peptide (1:10 ratio) were incubated using previously described assay conditions (20), except that the hydrolysis of peptide was monitored by reading the fluorescence polarization value of fluorescein on an Acquest machine (LJL Biosystem, Sunnyvale, Calif.) at an excitation wavelength of 485 nm and an emission wavelength of 530 nM. An in-house compound library was tested at 14.5 μM. For 50% inhibitory concentration (IC_{50}) determinations, compound concentrations ranging from 100 μM to 1 nM were used in the assay.

RESULTS

Effect of a penem inhibitor of SPase on growth of *E. coli* and *S. aureus*. The effect of penem on growth of *E. coli* TOP10 containing pMB2 was tested in the presence and absence of Pbn (Fig. 1). The addition of 100 μM penem to the culture medium caused a 31% reduction of the optical density. Almost complete growth inhibition was observed when both penem and Pbn were present. Results for growth inhibition by penem as a measure of optical density were corroborated by counts of viable cells. Pbn concentrations varying from 5 to 40 μg/ml were tested (data not shown). A concentration of 10 μg/ml was selected for further tests as the highest Pbn concentration that still presented a negligible effect on cell growth. *S. aureus* cultures were insensitive to penem concentrations up to 200 μM, in the presence or absence of Pbn (data not shown).

Construction of an *E. coli lepB* regulatable strain. PMB2 containing the *lepB* gene under the control of a repressible promoter was cloned into *E. coli*, and the chromosomal copy of *lepB* was replaced by a KAN resistance gene. *lepB* sequences flanking the *kan* gene allowed homologous recombination

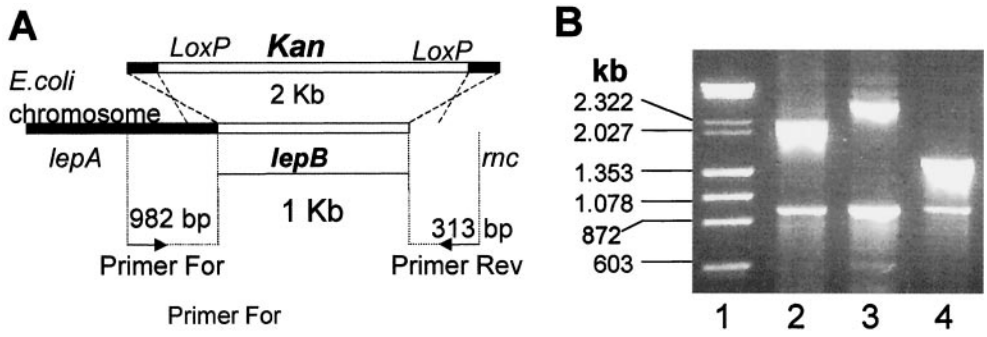

FIG. 2. Construction of a *lepB* regulatable *E. coli* strain. (A) Schematic representation of the strategy utilized to replace the *lepB* gene by a *loxP-Kan-loxP* resistance cassette. The positions of the flanking primers used to confirm the gene replacement are indicated. The figure is not drawn to scale. (B) PCR amplification of *E. coli* chromosomal DNA with Primer For and Primer Rev. Lanes: 1, molecular size markers; 2, parent strain *E. coli* 391; 3, KAN-resistant *E. coli lepB* regulatable strain; 4, KAN-sensitive *E. coli lepB* regulatable strain.

(Fig. 2A). Recombinants with the chromosomal copy of *lepB* deleted were selected as *E. coli* cells able to grow in the presence of Ara exclusively. Integration of the *kan* gene into the chromosome was monitored by PCR analysis (Fig. 2B). DNA fragments were amplified with primers designed according to the sequences of the *lepB* flanking genes. The fragment from the parental strain was smaller than the KAN-resistant *lepB* regulatable strain. Upon removal of the KAN marker, the size of the PCR fragment amplified decreased relative to the parental strain, and the resulting strain was unable to grow on medium with KAN or without Ara. The *lepB* regulatable strain was capable of growing on either RM medium or LB medium supplemented with Ara, but growth was slower with the former medium. In addition, a lower optical density was observed at the stationary phase following growth on RM medium (data not shown). Hence, LB medium was utilized for further studies.

Expression of *lepB* and its effect on growth inhibition by penem. Several Ara concentrations were evaluated for the induction of gene expression in the *lepB* regulatable strain of *E. coli* (Fig. 3A). The minimum Ara concentrations required to support growth similar to that of the parental strain after 24 h of incubation were in the range of 0.0002 to 0.0004%. Expression of the *lepB* gene was monitored by Western blot analysis (Fig. 3B). After incubation of the cells for 10 or 22 h with 0.05% Ara, the level of SPase in the *lepB* regulatable strain was similar to the level observed in cell extracts from the parental *E. coli*. Cell extracts from the *lepB* regulatable strain incubated with 0.0002% Ara had lower levels of SPase. Purified SPase was run in parallel. A second band observed with the purified enzyme represents an autolysis product (20). Some growth of the *lepB* regulatable strain in the absence of the inducer may be due to residual SPase in the cells. Hence, the inoculum was routinely prepared by growing the cells on plates containing 0.01% Ara. The levels of SPase in the inoculum prepared as described above were not sufficient to support residual growth in fresh medium without Ara.

The effect of an SPase inhibitor was tested utilizing cells producing various levels of the target enzyme (Fig. 4A). Under-expression of *lepB* sensitized the cells to penem, with com-

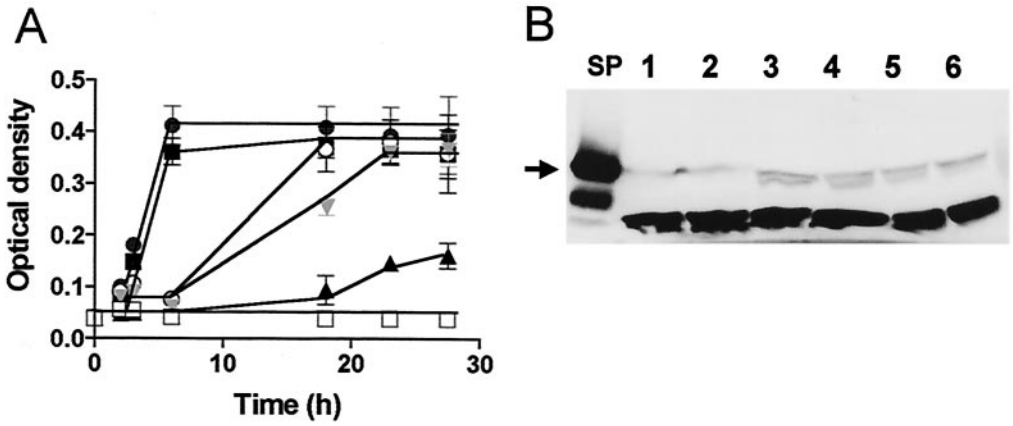

FIG. 3. Effect of L-Ara concentration on growth and SPase production by a KAN-sensitive *E. coli lepB* regulatable strain that contains pMB2. (A) Growth in the presence of various Ara concentrations (% [wt/vol]). Open squares, 0; closed triangles, 0.0001; inverted triangles, 0.0002; open circles, 0.0004; closed squares, 0.05; closed circles, 0.1. Experiments were performed in duplicate and repeated independently. (B) Western blot analysis of *lepB* expression after 10 h (lanes 1, 3, and 5) and 22 h (lanes 2, 4, and 6) of incubation. Three micrograms of total cell protein was loaded in each lane. Lanes: SP, purified SPase; 1 and 2, *lepB* regulatable strain with 0.0002% Ara; 3 and 4, *lepB* regulatable strain with 0.05% Ara; 5 and 6, parent strain.

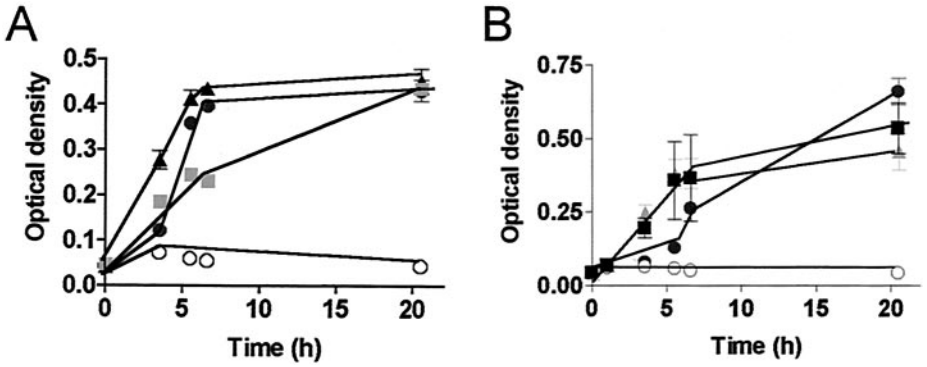

FIG. 4. Growth rescue of an *E. coli lepB* regulatable strain by increased expression of *lepB* in the presence of penem and different Ara concentrations. (A) Growth on LB medium. (B) Growth on LB medium supplemented with 10 μg of Pbn per ml. Closed squares, 0.0004% Ara; closed triangles, 0.05% Ara; open circles, 0.0004% Ara and 10 μM penem; closed circles, 0.05% Ara and 10 μM penem. Experiments were performed in duplicate and repeated independently.

plete growth inhibition observed at a concentration of 10 μM. The SPase produced in cells grown with 0.05% Ara was sufficient to support growth similar to that of the parental strain in both the presence and absence of penem. The same growth rescue by production of higher levels of SPase was observed when 10 μg of Pbn per ml was included in the medium (Fig. 4B).

SPase inhibitors from a high-throughput screen. A biochemical assay for SPase activity identified 112 compounds that inhibited SPase at 14.5 μM. IC_{50} values were determined for the 112 compounds, and three with antimicrobial activity (MD1, MD2, and MD3) were tested further in a cell-based assay. The structures of these SPase inhibitors are shown in Fig. 5. The IC_{50} values for SPase inhibition were 2.2, 5.3, 5.0, and 1.3 μM for MD1, MD2, MD3, and penem 69, respectively. MD1, MD2, and MD3 displayed antimicrobial activity against *S. aureus* but did not inhibit growth of wild-type *E. coli* (Table 1).

Synthesis of penem and MD4 and stability tests for SPase inhibitors. The penem 64 compound was synthesized as previously reported (6). MD4 is a reactive compound synthesized to test the effect of a possible breakdown product of MD3, one of the SPase inhibitors (Fig. 6). The IC_{50} value for SPase inhibition in the biochemical assay by MD4 was 4.9 μM. MD3 was very unstable, both in DMSO and Tris buffer. Degradation of this compound led to the formation of reactive products. MD2 was stable after 5 days in DMSO but appeared to undergo solvolysis in methanol, with approximately 30% conversion after 1 week. After 1 day of incubation in DMSO, the nuclear magnetic resonance spectrum of MD1 suggested that it was 25% degraded. Similar degradation results for the MD1 nuclear magnetic resonance spectrum was observed after additional incubation for 6 days.

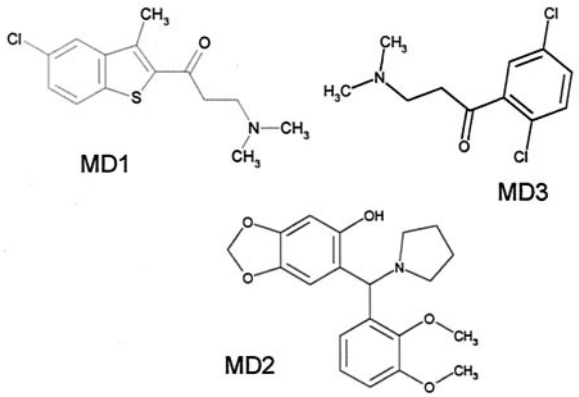

FIG. 5. Chemical structures of SPase inhibitors. MD1, MD2, and MD3 were selected from a high-throughput screen that utilized a biochemical assay.

In vivo inhibition of SPase. No growth inhibition of the *lepB* regulatable strain was observed in the presence of 30 μM MD1, MD2, or MD3 in LB medium containing 0.0004% Ara (data not shown). However, when the effect of these compounds was tested in the presence of 10 μg of Pbn per ml, growth inhibition and growth rescue were observed at low and high Ara concentrations, respectively (Fig. 7). When penem was tested at 30 μM, the growth rescue of the *lepB* regulatable strain with 0.05% Ara was delayed relative to growth with 10 μM penem and 0.05% Ara (Fig. 4A and Fig. 7A). MD4, a reactive analog of MD3, also inhibited growth of the *lepB* regulatable strain (Fig. 7A). However, growth rescue at a higher Ara concentration in the presence of both MD3 and MD4 took place only after a prolonged incubation time. Penem, MD3, and MD4 completely inhibited the growth of cells under-expressing *lepB*, while only partial inhibition was observed in the presence of MD1 and MD2.

TABLE 1. Susceptibility of a panel of microorganisms to SPase inhibitors

Microorganism	MIC (μg/ml)		
	MD1	MD2	MD3
E. coli ATCC 25922	>64	>11.4	>9
Haemophilus influenzae ATCC 49766	8	>11.4	9
Moraxella catarrhalis ATCC 25238	2	5.7	2.3
S. aureus ATCC 13709	2	11.4	4.5
Streptococcus pneumoniae ATCC 49619	ND[a]	11.4	>9
Candida albicans ATCC 90028	4	>11.4	9

[a] ND, not determined.

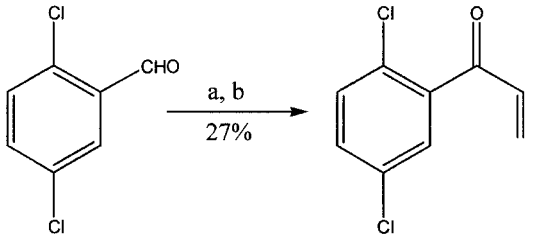

FIG. 6. Synthesis of MD4. a, vinylmagnesium bromide, THF, 0°C; b, MnO_2, CH_2Cl_2.

DISCUSSION

SPase is essential for bacterial growth (10). Under the conditions routinely utilized for inoculum preparation, no residual growth of the *lepB* regulatable strain in the absence of Ara was observed. However, some increase in the optical density following incubation in medium without Ara can be observed, presumably contingent upon the residual levels of SPase in the cells. This is in agreement with previous work aimed at demonstrating the essentiality of the *lepB* gene product (10). Those authors had observed that the growth rate of a *lepB* regulatable strain was identical in the presence or absence of Ara during the initial hours of incubation in liquid medium, a result that suggested that time was needed to dilute the SPase originally present among the daughter cells.

An *E. coli* recombinant strain was constructed, with *lepB* expression from pBAD dependent on the Ara concentration. Previous analyses of gene expression from plasmids containing the *araBAD* promoter have indicated that at subsaturating concentrations of Ara, intermediate levels of expression represent a population average of cells that are individually different (19). However, those authors used a very high Ara concentration (0.1%) to induce green fluorescent protein synthesis prior to examining the cells by fluorescence microscopy. We have observed that when the Ara concentration in the medium is lowered to the minimum sufficient to support growth similar to that of the parent strain, the cells become sensitized to inhibitors of SPase. An SPase inhibitor, penem (6), was utilized to validate the system. *E. coli* was sensitized to this compound in the presence of Pbn. Polymyxin derivatives that lack the fatty acid tail are generally not bactericidal but are capable of permeabilizing the outer membrane (22). In this respect, the best-characterized derivative is Pbn. Its MICs for *E. coli* and *Salmonella enterica* serovar Typhimurium are higher than 300 μg/ml, but even as low a concentration as 0.3 μg/ml is sufficient to permeabilize the outer membrane (22). In the present work Pbn itself had a negligible negative effect on cell growth at the concentrations utilized. The growth results obtained at different Ara concentrations and in the presence or absence of penem might be indicative that specific inhibition of the target activity in vivo was taking place. However, the use of other systems and/or promoters (19, 25) may provide more tightly regulated expression at the level of individual cells and increase the accuracy of the cell-based assays.

Three SPase inhibitors selected with a biochemical assay were active against bacterial strains, although they did not inhibit growth of wild-type *E. coli* at the concentrations tested. Those compounds affected growth of the *E. coli* strain underexpressing *lepB* only in the presence of Pbn, perhaps due to the *E. coli* permeability barrier. Cell-based assays employing gram-positive organisms might be more sensitive to lower concentrations of the compounds. MD3 was very unstable, and it is possible that a reactive degradation product was solely responsible for the inhibition of the SPase biochemical reaction as well as cell growth. MD4 is a reactive analog of MD3. MD3 and MD4 yielded similar inhibition profiles when tested with the cell-based assay and also had similar IC_{50} values for SPase inhibition with a biochemical assay. When either MD3 or MD4 was present in the medium, growth rescue by high concentrations of Ara was observed only after prolonged incubation. This is in contrast to the inhibition observed with penem, a time-dependent irreversible inhibitor of SPase (17). MD1 and MD2 only partially inhibited growth of the strain under-expressing *lepB*. The percentage of growth inhibition observed correlated with the IC_{50} values obtained with the biochemical assay, although the mechanism of action of these compounds in vivo is not clearly defined. Similar to the data obtained with penem, these results also indicate that a cell-based assay uti-

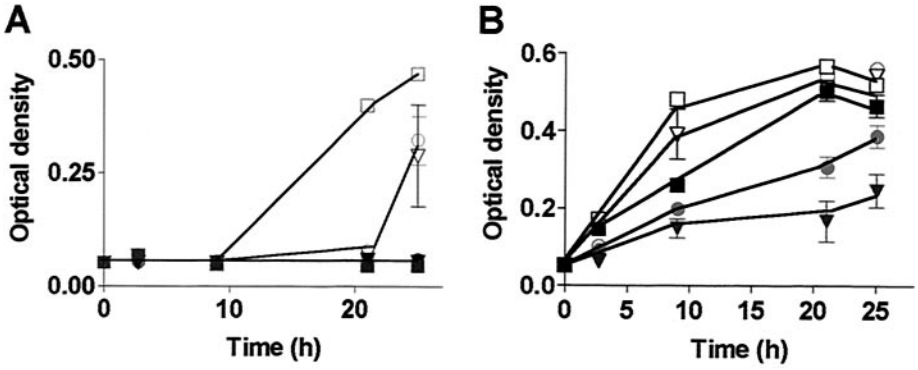

FIG. 7. Growth impairment of an *E. coli lepB* regulatable strain as an indication of SPase inhibition by 30 μM MD1, MD2, MD3, or MD4. The growth medium was LB containing 10 μg of Pbn per ml. Penem (30 μM) was included as a control for the experiments. The compounds were dissolved in 100% DMSO. The final DMSO concentration in the medium was 1%. Closed symbols, 0.0004% Ara; open symbols, 0.05% Ara. (A) Growth in the presence of MD3 and MD4. Squares, penem; inverted triangles, MD3; circles, MD4. (B) Growth in the presence of MD1 and MD2. Squares, DMSO; inverted triangles, MD1; circles, MD2. Experiments were done in triplicate and repeated twice.

lizing strains under-expressing a given gene may be used for initial investigations of target inhibition in whole cells.

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