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(54) Title: HIGH THROUGHPUT SCREEN FOR INHIBITORS OF THE FOLATE BIOSYNTHETIC PATHWAY IN BACTERIA

(57) Abstract: Methods for identifying compounds that are inhibitors of bacterial tetrahydrofolate biosynthesis are disclosed. Such compounds can be used as lead compounds in methods for preparing antibacterial agents for treating bacterial infections (e.g., in humans, animals, and plants). The disclosed methods allow for high throughput screening of libraries of test compounds.

HIGH THROUGHPUT SCREEN FOR INHIBITORS OF
THE FOLATE BIOSYNTHETIC PATHWAY IN BACTERIA

CROSS-REFERENCE TO RELATED APPLICATION

5 This application claims priority from U.S. provisional application Serial No. 60/224,925 filed on August 11, 2000, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

10 The invention relates to methods for identifying inhibitors of tetrahydrofolate biosynthesis in bacteria.

BACKGROUND OF THE INVENTION

15 Tetrahydrofolate is an essential cofactor for many biosynthetic enzymes. It acts as a carrier of one-carbon units in the syntheses of such critical metabolites as methionine, purines, glycine, pantothenate, and thymidylate. For example, the enzyme ketopantoate hydroxymethyl transferase, encoded by the *panB* gene, requires a tetrahydrofolate cofactor to synthesize precursors of pantothenate. As tetrahydrofolate is synthesized de novo in bacteria, inhibition of its synthesis kills cells. Indeed, two very effective antibiotics, sulfonamide and trimethoprim, kill bacterial cells by blocking tetrahydrofolate production. These two antibiotics, which are often used in combination with each other, are commonly prescribed
20 for the treatment of urinary tract infections, enteric infections such as shigellosis, and respiratory tract infections. The success of these drugs is indicative of the vulnerability of many pathogenic bacteria to inhibitors of tetrahydrofolate synthesis.

25 Bacteria have a multiple step pathway for the synthesis of the tetrahydrofolate cofactor. In one branch of the pathway, the metabolites chorismate and glutamine are substrates for aminodeoxychorismate synthase, encoded by the *B. subtilis* genes, *pabA* and *pabB*, which produces 4-amino 4-deoxychorismate. Aminodeoxychoismate lyase, encoded

by *B. subtilis pabC*, then converts 4-amino 4-deoxychorismate to *para*-aminobenzoic acid (PABA), an important precursor. In another branch, a number of enzymes, including those encoded by *B. subtilis mtrA*, *folA*, and *folK*, produce the precursor 2-amino-4-hydroxy-6-hydroxy methyl-7, 8-dihydropteridine diphosphate. This precursor and PABA are
5 substrates for dihydropteroate synthetase, encoded by the *B. subtilis sul* gene (homologous to the *E. coli dhps* and *folP* genes), which produces dihydropteroate. Sulfonamides, such as sulfamethoxazole, are competitive inhibitors of dihydropteroate synthase.

Dihydropteroate is modified by the bifunctional enzyme encoded by *B. subtilis folC* to produce dihydrofolate. Finally, DHFR (dihydrofolate reductase), encoded by *B. subtilis dfrA*, modifies this dihydrofolate to generate the end product tetrahydrofolate. Trimethoprim
10 is a competitive inhibitor of bacterial DHFRs. This selectivity is critical, as eukaryotic DHFRs are unimpeded by the antibiotic.

The emergence of antibiotic resistant strains of bacteria has heightened the urgency of developing new antibiotics. Indeed, resistance to sulfonamides and trimethoprim is prevalent
15 and spreading. For example, before 1983, trimethoprim resistance in *Shigella* was rare and occurred in less than 4% of cases. However, by the 1990s, the frequency of resistance in isolates was as much as 52% (Huovinen (1997) *Clinical Infectious Disease* 24 (Suppl. 1):S63-66). Similarly, as many as 42% of *Shigella* species are sulfonamide resistant (Huovinen, *supra*). Often resistance to these antibiotics is a plasmid borne trait that can be
20 transmitted horizontally to other bacterial species.

SUMMARY OF THE INVENTION

The invention is based upon the discovery that the activity of promoters of certain genes is increased in the presence of compounds that inhibit *B. subtilis* tetrahydrofolate
25 biosynthesis. Thus, compounds that inhibit tetrahydrofolate biosynthesis can be identified by their ability to increase the activity of the *B. subtilis panB* promoter. Various promoters can be used in the invention, provided that the activity of the promoter is upregulated by a tetrahydrofolate biosynthesis inhibitor, such as trimethoprim or sulfonamide. Tetrahydrofolate biosynthesis inhibitors that slow the growth of, or kill, bacteria are
30 candidate antibacterial agents that can be used in methods of treating bacterial infections.

The invention thus provides a rapid and convenient method for identifying (i) compounds that inhibit tetrahydrofolate biosynthesis and which can subsequently be derivatized to produce antibacterial agents, as well as (ii) compounds that inhibit tetrahydrofolate biosynthesis and which are antibacterial agents. If desired, such inhibitors of tetrahydrofolate synthesis can be further derivatized using standard medicinal chemistry techniques to produce anti-microbials of increased potency.

Accordingly, the invention features a method for determining whether a test compound is an inhibitor of bacterial tetrahydrofolate biosynthesis. The method includes: (i) contacting a bacterial cell with a test compound, wherein the bacterial cell contains (α) a promoter (e.g., *panB*), the activity of which is increased in the presence of a compound that inhibits tetrahydrofolate biosynthesis, operably linked to (β) a reporter gene; and (ii) measuring activity of the promoter, wherein an increase in activity, relative to the level of activity of the promoter in the absence of the test compound, indicates that the test compound is an inhibitor of bacterial tetrahydrofolate biosynthesis.

The invention also includes a method for determining whether a test compound is an antibacterial agent, the method comprising: (i) contacting a bacterial cell with a test compound, wherein the bacterial cell contains (α) a promoter (e.g., *panB*), the activity of which is increased in the presence of a compound that inhibits tetrahydrofolate biosynthesis, operably linked to (β) a reporter gene; (ii) measuring activity of the promoter, wherein an increase in activity, relative to the level of activity of the promoter in the absence of the test compound, indicates that the test compound is an inhibitor of tetrahydrofolate biosynthesis; and (iii) determining whether the compound is an antibacterial agent by determining whether the compound kills, or slows the growth of, bacteria. Optionally, the test compound may be further assayed in a biochemical assay (e.g., in an extract of the cell) to determine which step in the pathway is inhibited, and to confirm that the test compound inhibits tetrahydrofolate biosynthesis. For example, inhibition of tetrahydrofolate biosynthesis can be detected as inhibition of incorporation of *para*-aminobenzoic acid (PABA) into cells. Conventional methods can be used to measure inhibition of incorporation of PABA into cells (Herrington (1994) *Anal Biochem* 216:427-430). Other optional tests can be incorporated into the method. For example, growth inhibition and antibacterial effect of the test compound can be assayed. The assay can be a plate assay or a liquid culture assay in a microplate. The assay

can be performed with *B. subtilis* cells, but also with any Gram-positive or Gram-negative bacterial strain that grows in culture.

An increase in activity of the promoter can be measured, for example, by measuring expression of a reporter gene that is operably linked to the promoter, such as a *lacZ*, *cat*, *gus*,
5 a green fluorescent protein gene, or a luciferase gene. Other suitable reporter genes are well known in the art and can be used in the invention. If desired, the activity of the promoter can be measured by measuring binding of antibodies to a product of the reporter gene (e.g., a protein encoded by the reporter gene), with an increase in the level of bound antibodies reflecting an increase in activity of the promoter. Alternatively, activity can be measured by
10 measuring the level of mRNA transcribed from the reporter gene, with an increase in the mRNA level reflecting an increase in promoter activity. Further, the expression of the *panB*, *panC*, and *panD* genes can be measured by determining the levels of the panB, panC, and panD polypeptides, for example, using antibodies specific to the polypeptides. Alternatively, enzymatic assays can be performed on crude cell extracts for aspartate 1-decarboxylase
15 activity (panD), pantothenate synthase activity (panC), and ketopantoate hydroxymethyltransferase activity (panB) (see Cronan *et al.* (1982) *J Bact* 149:916-922).

The invention also provides methods of preparing (i) an inhibitor of tetrahydrofolate biosynthesis and/or (ii) an antibacterial agent. The methods include: screening multiple test
20 compounds by the methods described above; identifying candidate compounds that upregulate promoter activity; isolating one or more lead compounds from the candidate compounds; identifying and selecting a lead compound that inhibits tetrahydrofolate biosynthesis or bacterial growth; and formulating the selected lead compound as an inhibitor of tetrahydrofolate biosynthesis or as an antibacterial agent. A "lead compound" is a test
25 compound that increases promoter activity to a level at least three times the standard deviation above the mean of a group of non-active compounds or controls. If desired, lead compounds can be subsequently derivatized using conventional medicinal chemistry methods, as described herein.

Similarly, the invention features methods for preparing (i) an inhibitor of tetrahydrofolate biosynthesis or (ii) an antibacterial agent. The methods include screening
30 multiple test compounds by the methods described above; identifying candidate compounds that upregulate promoter activity; isolating one or more lead compounds from the candidate compounds; derivatizing the lead compound(s), thereby producing a derivative of the lead

compound; identifying derivatives that inhibit tetrahydrofolate biosynthesis or bacterial growth; and formulating the derivative as an inhibitor of tetrahydrofolate biosynthesis or as an antibacterial agent (e.g., by admixture with a pharmaceutically acceptable carrier).

Inhibitors of tetrahydrofolate biosynthesis and antibacterial agents prepared by such methods also are included within the invention. Such compounds can be used in methods for inhibiting bacterial tetrahydrofolate biosynthesis or growth of bacteria in an organism having a bacterial infection.

As used herein, the term "operably linked" requires that a nucleic acid sequence is connected to a promoter sequence in a manner, which allows for transcriptional expression of the nucleotide sequence in vivo.

The invention offers several advantages. For example, various embodiments of the invention can readily be used for high-throughput screening (HTS) of a wide variety of test compounds. Thus, lead compounds can readily be selected from a large number of test compounds. Assays employing the *panB* promoter are capable of detecting tetrahydrofolate biosynthesis inhibitors at concentrations both above and below their minimal inhibitory concentration (MIC). Thus, the assays described herein provide a high level of sensitivity and are expected to detect growth inhibitory (i.e., bacteriocidal) compounds, as well as less potent inhibitors of the tetrahydrofolate biosynthesis pathway, which can be subsequently modified using standard medicinal chemistry techniques and by evaluating structure-activity relationship (SAR) data. Because the assays are cell-based, they can be used to identify antibacterial agents that can efficiently enter bacterial cells. Thus, the assays enable the identification of potent antibacterial compounds and compounds of structural interest that may have relatively modest potency, but have favorable cell permeability properties. In addition, because tetrahydrofolate is the product of a multi-step biochemical pathway, the methods disclosed herein enable the identification of compounds that may inhibit any enzymatic function or step in the pathway.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, technical manuals, and other references mentioned herein are incorporated by reference in their entirety. In case of

conflict, the present application, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of the bacterial tetrahydrofolate biosynthesis pathway. The targets of the tetrahydrofolate biosynthesis inhibitors, sulfamethoxazole and trimethoprim, are shown.

Fig. 2 is a map of the pDG268 vector.

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Fig. 3 is a histogram depicting the level of β -galactosidase induction of a plasmid containing the *panB* promoter operably linked to the *lacZ* gene after 5 hours of exposure to 4 $\mu\text{g/ml}$, 2 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$, and 0.25 $\mu\text{g/ml}$ of trimethoprim (with drug) in comparison to a mock treated control (no drug).

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Fig. 4 is a histogram depicting the level of β -galactosidase induction of a plasmid containing the *panB* promoter operably linked to the *lacZ* gene after 5 hours of exposure to 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.13 mg/ml of sulfamethoxazole (with drug) in comparison to a mock treated control (no drug).

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DETAILED DESCRIPTION

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The invention provides methods for determining whether a test compound is an inhibitor of bacterial tetrahydrofolate biosynthesis. The invention derives from the discovery that compounds that inhibit tetrahydrofolate biosynthesis cause an increase in the expression of the *panBCD* operon. As discussed in detail below, the activity of the *panB* promoter is increased upon inhibition of the tetrahydrofolate biosynthesis pathway. Generally, the methods of the invention involve determining whether the test compound induces an increase in activity in the *panB* promoter, as indicated by an increase in expression of a reporter gene operably linked to the promoter (i.e., a reporter-based screen). Thus, compounds that increase the activity of the *panB* promoter can be expected to inhibit bacterial tetrahydrofolate biosynthesis. Optionally, the test compound can be further tested to confirm

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that it inhibits tetrahydrofolate biosynthesis (i.e., in a biochemical screen). These methods are described in further detail below.

Part I: Reporter-Based Screens

5 Genetic Constructs: Conventional transcriptional profiling methods can be used to identify promoters that have increased activity in the presence of antibacterial agents such as trimethoprim and sulfamethoxazole, which are inhibitors of tetrahydrofolate biosynthesis. In such an assay, certain bacterial promoters display an increase in activity in the presence of antibacterial agents, as evidenced by an increase in the level of mRNA transcripts of the
10 sequences.

 A "promoter" is a minimal sequence sufficient to direct transcription; the promoter is located in the 5' region of a native gene. A sequence containing the *panB* promoter is set forth as SEQ ID NO:1. GenBank entry Accession No. L47709 contains a listing of the sequence of the *B. subtilis panB* operon as well as flanking sequences. The *panB* promoter
15 can be found in the region of about nucleotide 13043 to about 13536 in the GenBank entry L47709.

 To confirm that the activity of a promoter is increased in the presence of an antibacterial agent, each promoter can be operably linked to the coding sequence of a reporter gene, such as the *E. coli lacZ* gene. The resulting genetic constructs then are
20 inserted into a plasmid, which is stably propagated, e.g., in *B. subtilis*. Such strains are treated with an antibacterial agent, such as trimethoprim and sulfamethoxazole, and the level of promoter activity, as measured by *lacZ* expression is measured. An increase in the level of promoter activity, relative to the level of *lacZ* expression in untreated, control cells, confirms that promoter activity is modulated by the antibacterial agent.

25 The "reporter gene" can be any sequence the expression of which can be detected or measured, other than the coding sequence to which the promoter naturally is operably linked. Typically, the reporter gene is heterologous to the bacterial strain in which promoter activity is measured. Examples of suitable reporter genes include, without limitation, *lacZ*, the bacterial chloramphenicol acetyltransferase (*cat*) gene, luciferase genes, the bacterial *gus*
30 gene, and the like. Also included are sequences that encode fluorescent markers, such as green fluorescent protein (GFP). The aforementioned reporter genes, and methods for measuring their expression, are well known in the art.

Bacterial Strains: *B. subtilis* strains having the *panB* promoter operably linked to the coding sequence of a reporter gene can be used to assay the ability of test compounds to increase activity of the *panB* promoter. If desired, the promoter and reporter gene can be stably integrated into the bacterial chromosome. Alternatively, the *panB* promoter and coding sequence of the reporter gene can be located on a plasmid that is introduced into a bacterium (e.g., *E. coli*).

Test Compounds: The "test compound" can be any compound, such as a small organic or inorganic molecule, amino acid, polypeptide, nucleic acid, peptide nucleic acid, carbohydrate, or polysaccharide. The test compounds can be synthetic, naturally occurring, or a combination of synthetic and natural components. If desired, the test compound can be a member of a library of test compounds (e.g., a combinatorial chemical library) or a component of a cellular extract or bodily fluid (e.g., urine, blood, tears, sweat, or saliva).

Test compounds that increase the activity of the promoter, relative to the level of promoter activity in the absence of the test compound, are considered inhibitors of bacterial tetrahydrofolate biosynthesis. The level of promoter activity measured in the presence of the test compound then is compared with the level measured in the absence of the test compound. Generally, an increase in the level of promoter activity that is at least 3 times the standard deviation of a test compound set (i.e., a set of one or more test compounds), plus the mean of a test compound set, relative to the level of gene expression in the absence of the test compound, indicates that the test compound is an inhibitor of tetrahydrofolate biosynthesis. Such an increase may be, for example, 5-fold, 10-fold, 20-fold, 50-fold, or even 100-fold. A relatively high level of induction generally indicates that the test compound has a relatively high level of potency.

Promoter activity, as measured by reporter gene expression, can be measured by any of a number of conventional methods, and the optimal method will depend upon factors such as the nature and function of the reporter gene. In general, suitable assays of reporter gene expression include methods such as (i) assaying the function of a product of the reporter gene (e.g., measuring an enzymatic reaction catalyzed by a product of the reporter gene); (ii) measuring the level of protein expressed from the reporter gene (e.g., by SDS-PAGE or in an immunoassay using antibodies (e.g., polyclonal or monoclonal antibodies) that specifically bind to the product of the reporter gene; and (iii) measuring the level of mRNA transcribed

from the reporter gene. Included within the invention are assays that permit high throughput screening of test compounds.

The assays of promoter induction can be carried out in virtually any reaction vessel or receptacle. Examples of suitable receptacles include 96-well plates, 384-well plates, test tubes, centrifuge tubes, and microcentrifuge tubes. The methods can also be carried out on surfaces such as metal, glass, ceramics, paper, polymeric chips, membrane surfaces, resins, or the surface of a matrix-assisted laser-desorption ionization mass spectrometry (MALDI-MS) plate.

10 Part II: Biochemical Screen

Once a test compound is identified as an inhibitor of tetrahydrofolate biosynthesis using the above-described reporter-based assay, the compound is thus a potential or candidate anti-bacterial agent. Candidate compounds can be further tested with a simple cell-based assay, or if desired, in a biochemical assay. One simple cell-based assay is the uptake by cells of radio-labeled PABA from the media. Herrington (1994) *Anal Biochem* 216:427-430 described such an assay using ¹⁴C-PABA and demonstrated that PABA uptake is inhibited by treatment with trimethoprim and sulfathiazole. The details of this assay are set forth below.

Candidate compounds identified in the above-described screen can be tested in an in vitro biochemical assay for their ability to inhibit an enzymatic step in the synthesis of tetrahydrofolate. Candidate compounds, which are effective inhibitors in a biochemical assay, can be considered "lead" compounds or may be antibacterial agents.

For example, candidate compounds can be tested for their ability to inhibit enzymes encoded by tetrahydrofolate biosynthesis genes, such as *B. subtilis mtrA*, *folA*, *folK*, *sul*, *pabA*, *pabB*, *pabC*, *folC*, and *dfrA*. Various methods for measuring inhibition of steps in tetrahydrofolate biosynthesis are known in the art, and can be used in this aspect of the invention (see, e.g., Nar *et al.* (1995) *Proc Natl Acad Sci USA* 92:12120-25; Zimmerman *et al.* (1977) *J Med Chem* 20:1213-15; Bock *et al.* *Anal Biochem* (1978) 86:238-51; Swedberg *et al.* (1979) *J Bact* 137:129-136; Viswanathan *et al.* (1995) *J Bact* 177:5918-5923; Bognar *et al.* (1985) *J Biol Chem* 1985;260:5625-30; Myoda *et al.* (1984) *Gene* 29:139-147). Table 1 sets forth various *B. subtilis* and *E. coli* tetrahydrofolate biosynthesis genes and enzymes, along with the enzymatic reactions that the enzymes catalyze. Standard assays for measuring

inhibition of the enzymatic reactions are summarized in Table 1 and can be used in the invention.

Table 1: Assays of Tetrahydrofolate Biosynthesis Enzymes

Enzyme Activity	Gene Name	Reaction Catalyzed by the Enzyme	Assay	Reference
GTP cyclohydrazase I	<i>mtrA</i>	GTP ↔ 7,8 dihydroneopterin triphosphate	measure neopterin fluorometrically (excite 365 nm, emission 446 nm) after reverse-phase HPLC	Nar <i>et al.</i> (1995) <i>Proc Natl Acad Sci USA</i> 92:12120-25.
7,8 dihydroneopterin aldolase (DHNA)	<i>folA</i>	7,8 dihydroneopterin ↔ 6-hydroxymethyl-7,8-dihydropterin + glycoaldehyde	Production of [¹⁴ C]glycoaldehyde from dihydroneopterin	Zimmerman <i>et al.</i> (1977) <i>J Med Chem</i> 20:1213-15
6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK)	<i>folK</i>	ATP + 6-hydroxymethyl-7,8-dihydropterin ↔ AMP + 6-hydroxymethyl-7,8-dihydropterin pyrophosphate	Thin-layer chromatography of reaction products	Bock <i>et al.</i> (1978) <i>Anal Biochem</i> 86:238-51
Dihydropteroate Synthase (DHPS)	<i>sul/folP/dhps</i>	PABA ↔ dihydroxypteroate	Incorporation of [¹⁴ C]PABA into dihydropteroic acid	Swedberg <i>et al.</i> (1979) <i>J Bact</i> 137:129-136.
aminodeoxychorismate synthase	<i>pabAB</i>	chorisimate + glutamine ↔ 4-amino 4-deoxychorismate	Assay PABA production fluorimetrically from glutamine & chorismate	Viswanathan <i>et al.</i> (1995) <i>J Bact</i> 177:5918-5923
aminodeoxychorismate lyase	<i>pabC</i>	4-amino 4-deoxychorismate ↔ PABA	Assay PABA production fluorimetrically	Viswanathan <i>et al.</i> (1995) <i>J Bact</i> 177:5918-5923
Dihydrofolate:folylpolyglutamate synthetase	<i>folC</i>	dihydropteroate + ATP + glutamate ↔ dihydrofolate + ADP + Pi	Incorporation of [¹⁴ C] glutamate into folate products.	Bognar <i>et al.</i> (1985) <i>J Biol Chem</i> 1985;260:5625-30
Dihydrofolate reductase	<i>dfrA</i>	dihydrofolate + NADPH + H ⁺ ↔ tetrahydrofolate + NADP	Assay reduction of dihydrofolate spectrophotometrically at 340 nm.	Myoda <i>et al.</i> (1984) <i>Gene</i> 29:139-147.

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If desired, inhibition of tetrahydrofolate biosynthesis can be measured in a cell extract that contains all of the components (e.g., enzymes, cofactors, carrier molecules, and buffers) normally necessary for a particular step that is needed in the synthesis of folates. Cell

extracts containing enzymes, cofactors, and carrier molecules can be cytoplasmic, cytosolic, or membrane preparations, whole cells, or naturally occurring or synthetic mixtures composed of natural or unnatural components, or both. Carrier molecules included in the cell extract can include numerous components, such as molecular transport machinery and membranes. The substrate for the reaction can be contained within the cell extract initially, it can be added in solution, e.g., as a dry or liquid additive, or it can be generated *in situ* (e.g., as the product of another reaction). The substrate can be detectably labeled with a tag, for example, a radiolabel, a fluorescent label, a magnetic label, or as a biotinylated derivative.

After incubation of the cell extract/substrate mixture under conditions that normally allow the particular step(s) to proceed, the mixture is assayed to determine whether the substrate remains and/or whether the corresponding product or products have been formed. The optimal duration of incubation varies with the particular synthesis step(s) being carried out and also with incubation temperature (e.g., at least 30 minutes, 1 hour, 2 hours, 3 hours, 5 hours, 8 hours, 12 hours, or longer, at, e.g., room temperature or lower, 30°C, 37°C, or higher, depending on the strain of bacteria).

Medicinal Chemistry

Once a compound has been identified as a lead compound for inhibiting tetrahydrofolate biosynthesis, principles of standard medicinal chemistry can be used to produce derivatives of the compound. Derivatives can be screened for improved pharmacological properties, for example, efficacy, pharmacokinetics, mammalian toxicity, stability, solubility, and clearance. The moieties that are responsible for the compound's activity can be revealed by examining its structure-activity relationships (SAR). Specifically, a person of ordinary skill in the art of chemistry could modify a moiety of the compound to study the effects of the modification on the potency of the compound and thereby produce derivatives of the compound having increased potency (*See*, e.g., Nagarajan et al., *Antibiot.* 41:430-438). For example, chemical modifications such as N-acylation, esterification, hydroxylation, alkylation, amination, amidation, oxidation, or reduction can be made. Such chemical modifications can be made according to conventional methods (*See*, e.g., Wade, *Organic Chemistry*, Prentice-Hall, Inc., New Jersey, 1987). In addition, structural information can be used to design and optimize derivatives of the inhibitor by using molecular modeling software and conventional methods. Molecular modeling software is

commercially available (e.g., from Tripos Inc., Molecular Simulations, Inc., and MDL Information Systems, Inc).

Use of Tetrahydrofolate Biosynthesis Inhibitors

5 A compound identified as a tetrahydrofolate biosynthesis inhibitor and/or as an antibacterial agent can be used to treat a bacterial infection in an organism (e.g., a plant or a mammal (e.g., a human, dog, cat, or cow)). Because tetrahydrofolate biosynthesis genes are widely conserved, inhibitors of tetrahydrofolate biosynthesis are expected to be useful in inhibiting tetrahydrofolate biosynthesis in a wide spectrum of bacteria. For example, the
10 compounds can be used to treat infections of gram-positive bacteria, particularly pathogenic bacteria, such as *Streptococcus* species, *Staphylococcus* species, and *Clostridia* species. Thus, the compounds can be used, for example, to treat infections caused by *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus endocarditis*, *Streptococcus faecium*, *Streptococcus sanguis*, *Streptococcus viridans*, or *Streptococcus hemolyticus*. In other applications, the compounds can be used to treat infections of gram
15 negative bacteria, e.g., *Shigella*, *E. coli*, *Klebsiella*, *Yersinia*, and *H. influenzae*.

 A composition containing an effective amount of a tetrahydrofolate biosynthesis inhibitor or an antibacterial agent can be administered (e.g., topically, orally, nasally, buccally, subcutaneously, or intraperitoneally) to an organism in a method of treatment.
20 Treatment typically includes administering an effective amount of a composition containing a tetrahydrofolate biosynthesis inhibitor or an antibacterial agent to a subject in need of such treatment, thereby inhibiting bacterial growth in the subject. Such a composition typically contains from about 0.1 to 90% by weight (e.g., 1 to 20% or 1 to 10%) of the antibacterial agent of the invention in a pharmaceutically acceptable carrier.

25 Solid formulations of the compositions for oral administration may contain suitable carriers or excipients such gelatin, lactose, acacia, sucrose, kaolin, mannitol, dicalcium phosphate, calcium carbonate, sodium chloride, or alginic acid. Disintegrators that can be used include, without limitation, micro-crystalline cellulose, corn starch, sodium starch glycolate and alginic acid. Tablet binders that may be used include acacia, methylcellulose,
30 sodium carboxymethylcellulose, polyvinylpyrrolidone (Povidone™), hydroxypropyl methylcellulose, sucrose, starch, and ethylcellulose. Lubricants that may be used include magnesium stearates, stearic acid, silicone fluid, talc, waxes, oils, and colloidal silica.

Liquid formulations of the compositions for oral administration prepared in water or other aqueous vehicles may contain various suspending agents such as methylcellulose, alginates, tragacanth, pectin, kelgin, carrageenan, acacia, polyvinylpyrrolidone, and polyvinyl alcohol. The liquid formulations may also include solutions, emulsions, syrups and elixirs containing, together with the active compound(s), wetting agents, sweeteners, and coloring and flavoring agents. Various liquid and powder formulations can be prepared by conventional methods for inhalation into the lungs of the mammal to be treated.

Injectable formulations of the compositions may contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene glycol, and the like). For intravenous injections, water soluble versions of the compounds may be administered by the drip method, whereby a composition containing the compound and a physiologically acceptable excipient is infused. Physiologically acceptable excipients may include, for example, 5% dextrose, 0.9% saline, Ringer's solution or other suitable excipients. For intramuscular preparations, a sterile formulation of a suitable soluble salt form of the compounds can be dissolved and administered in a pharmaceutical excipient such as Water-for-Injection, 0.9% saline, or 5% glucose solution. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, such as an ester of a long chain fatty acid (e.g., ethyl oleate).

A topical semi-solid ointment formulation typically contains a concentration of the active ingredient from about 0.1 to 20% wt/vol (e.g., 0.1 to 2% wt/vol of essentially pure material) in a carrier such as a pharmaceutical cream base. Various formulations for topical use include drops, tinctures, lotions, creams, solutions, and ointments containing the active ingredient and various supports and vehicles.

The optimal percentage of the active ingredient in each composition varies according to the formulation itself and the therapeutic effect desired. Appropriate dosages can be readily determined by those of ordinary skill in the art by monitoring an organism for signs of disease amelioration or inhibition, and increasing or decreasing the dosage and/or frequency of treatment as desired. The optimal amount of the composition used for treatment of conditions caused by or contributed to by bacterial infection may depend upon the manner of administration, the age and the body weight of the subject and the condition of the subject

to be treated. Generally, the antibacterial agent is administered to a subject at a dosage of 1 to 100 mg/kg of body weight (e.g., at a dosage of 1 to 10 mg/kg of body weight).

EXAMPLES

5 The invention is further described by the following examples, which are not intended to limit the scope of the invention.

Example 1: Identification of the *panB* Promoter

10 Conventional RNA profiling studies revealed that bacterial cells treated with the tetrahydrofolate biosynthesis pathway inhibitors trimethoprim (0.2 µg/ml or 10 µg/ml) and sulfamethoxazole (0.5 mg/ml or 2 mg/ml) in LB medium at 37°C for about 40, 60, 80, or about 100 minutes displayed an increase in levels of mRNA derived from the *panB*, *panC*, and *panD* genes. After 90 minutes of treatment with 10 µg/ml trimethoprim, *panB* mRNA levels were induced 10 fold, *panC* 54 fold, and *panD* 38 fold. Similarly, after 80 minutes of
15 treatment with 0.5 mg/ml sulfamethoxazole, *panD* mRNA levels were induced 148 fold. *panB*, *panC*, and *panD* are located in close proximity to each other, and all are oriented in the same direction as described in Merkel and Nichols, (1996) *FEMS Microbiology Letters* 143:247-252). *panB*, *panC*, and *panD* are expected to be transcribed in a single mRNA from the *panBCD* operon and hence to share the *panB* promoter. Transcriptional
20 profiling indicated that the *panB* promoter is upregulated by antibacterial agents trimethoprim and sulfamethoxazole.

B. subtilis strains containing a *panB* promoter (along with an insignificant portion of the coding sequence) operably linked to the coding sequence of an *E. coli lacZ* reporter gene were used to confirm that the activity of the promoter is upregulated by an inhibitor of
25 tetrahydrofolate biosynthesis. The sequence of the *panB* promoter (SEQ ID NO:1:

 “5’GCTAATGT GTTGGTACAA GCCCGTTGAT TTTGGTATAC TTCCATTGGG
CAGTATCGCC TGCGAACTGC ACCTATTATT AAAATAGATA GACATTGCAG
CAGTCTGCCT TGATCCAAA AAGGACTGGG ACAGAGGGAT GAAACTCGCC
GAACTTTAGA AAGTGAAGAA TCCTTCTCGT TGTAACGGAA GGTTTTTTTGG
30 CTTGCAGAAG AAAACGGCAG ATCATCTCCT CTAAACATGA GGAGGAGAAA
ACATGAAAAC AAAACTGGAT TTTCTAAAAA TGAAGGAGTC TGAAGAACCG
ATTGTCATGC TGACCGCTTA TGATTATCCG GCAGCTAAAC TTGCTGAACA
AGCGGGAGTT GACATGATTT TAGTCGGTGA TTCACTTGGG ATGGTTCGTCC

TCGGCCTTGA TTCAACTGTC GGTGTGACAG TTGCGGACAT GATCCATCAT
 ACAAAGCCG TTAAAAGGGG TGCGCCGAAT ACCTTTATTG TGACAG”) is described in
 GenBank Accession Number L47709. The promoter was isolated by PCR amplification of *B.*
subtilis genomic DNA using the primers: Ppan-EcoRI-up (5’ “CCG GAA TTC GCT AAT
 5 GTG TTG GTA CAA GCC CG”; SEQ ID NO:2) and Ppan-BamHI-low (5’ “CGC GGA
 TCC CAA TAA AGG TAT TCG GCG CAC CC”; SEQ ID NO:3).

The reporter strains used in these assays were constructed as follows. PCR products
 containing the promoters were digested with *EcoRI* and *BamHI*, and cloned into the multiple
 cloning site of pDG268, which also had been digested with *EcoRI* and *BamHI*. The plasmid
 10 pDG268 is a derivative of pGD364 (See, Cutting and Van der Horn, *Molecular Biological
 Methods for Bacillus*, eds. C.R. Harwood and S.M. Cutting, 1990, John Wiley and Sons, NY,
 NY, pp. 52-54). A schematic representation of pDG268 is set forth in Fig. 2. The plasmid
 pDG268 is able to replicate in *E. coli*, contains an ampicillin-resistance gene, and encodes a
 promoter-less *lacZ* coding sequence. The multiple cloning site (MCS) is positioned such that
 15 a promoter of interest can be directionally cloned to drive expression of the *lacZ* sequence.
 Adjacent to the MCS, and opposite the *lacZ* sequence, is a gene encoding chloramphenicol
 (Cm) resistance for *B. subtilis*. The entire Cm-MCS-*lacZ* region is flanked by the upstream
 and downstream section of the *B. subtilis amyE* gene (a “silent” locus in the *B. subtilis*
 chromosome). Thus, the promoter fusion of interest can be stably integrated by homologous
 20 recombination at the *amyE* site of the *B. subtilis* chromosome by transforming *B. subtilis*
 cells with the linearized plasmid, and selecting for resistance to chloramphenicol. The
 promoter fusions were placed at the *amyE* chromosomal location of the *B. subtilis* strain
 PY79, which is a wild-type *B. subtilis* strain that is devoid of prophages (Youngman et al.,
 1984, *Mol. Gen. Genet.* 195:424-433), resulting in strain PY79(*amy::PpanB::lacZ, cat*).

25 The reporter strains were incubated, separately, with trimethoprim (Fig. 3) or
 sulfamethoxazole (Fig. 4) at various concentrations, as shown by the different bars of the
 histogram. The strain PY79(*amy::PpanB::lacZ, cat*) and an antibacterial agent were
 incubated in a 96-well microplate for 5 hours. β -galactosidase activity was detected using
 the Tropix Galacton Star™ chemiluminescent substrate (3-chloro-5-(4-methoxyspiro{1,2-
 30 dioxetane-3,2'-(4'chloro)-tricyclo-[3.3.1.1^{3,7}] decan}-4-yl)phenyl-B-D-galactopyranoside)
 and the detection method described below. The fold induction of promoter activity was

calculated as the level of activity in the samples containing the antibacterial agent divided by the level of activity in the samples that lacked the agent.

The minimum inhibitory concentrations (MIC) for trimethoprim and sulfamethoxazole were determined for each of the reporter strains. To determine the MIC, a compound of interest (e.g., trimethoprim or sulfamethoxazole) was placed in a 96-well microtiter plate such that the concentration of the compound varies in various wells of the plate (e.g., by using a series of 2-fold dilutions of the compound). Approximately 5,000 cells from a logarithmically-growing culture (e.g., the *B. subtilis* strain PY79) in Luria broth were added to each well (e.g., in a volume of 50-80 μ l). The plate then was incubated at 37°C for approximately 18 hours. The OD₆₀₀ was then read to measure cell growth in each well of the plate. The lowest concentration of the compound that leads to complete killing of the culture (i.e., the concentration of compound for which the OD₆₀₀ nm is equal to a control well that contains only media and the test compound) is deemed the MIC for the particular compound and bacterial strain. The MICs for trimethoprim and sulfamethoxazole were 3 μ g/ml and 500 μ g/ml, respectively, in the reporter strain.

Example 2: Assay for Inhibitors of Tetrahydrofolate Biosynthesis

Having determined that antibacterial agents such as trimethoprim and sulfamethoxazole, increase the activity of the *panB* promoter, bacterial strains containing this promoter operably linked to a reporter gene (e.g., *lacZ*) can be used in a method for determining whether a test compound is an inhibitor of tetrahydrofolate biosynthesis. The following example of such a method describes use of the *panB* promoter in an initial assay. Comparable reagents, apparatuses, and methods can readily be substituted for those described herein.

Bacterial Strains: The bacterial strain PY79 (*amy::PpanB::lacZ, cat*), which contains the *panB* promoter operably linked to the coding sequence of a *lacZ* reporter gene, was used in the initial screen for inhibitors of tetrahydrofolate biosynthesis. To prepare a bacterial glycerol stock, the cell culture was mixed at a 1:1 ratio with sterile 50% glycerol, and 1.0 ml aliquots of the suspension were stored in cryovials at -80°C.

To prepare cells for use in the assay of test compounds, 5 μ l of the glycerol stock is diluted in 80 ml of low salt LB medium (LB broth containing 5 g/L of NaCl). A 15 μ l aliquot of this diluted glycerol stock is then added to 900 ml of low salt LB medium and

grown to an OD₆₀₀ of 0.6 in a shaking incubator at 30°C. Generally, a culture having an OD₆₀₀ of 0.4 to 0.6 is suitable for use in the screening assays.

Screening Plates: In this example, chemical libraries containing test compounds at 10 mg/ml in DMSO are diluted to 100 µg/ml with 50 mM HEPES buffer (pH 7.4). The test
5 compounds (e.g., 0.1 µg or 0.05 µg) are dispensed into each well of a microplate (e.g., a 96- or 384-well microplate). The dispensed compounds are dried onto the plates (e.g., by leaving the plates in a fume hood overnight), and the plates are stored in the freezer and then brought to room temperature before being used in the screening assay.

A 50 µl sample of the bacteria is added to each well of the microplate. Trimethoprim
10 is used as a control in wells of the microplate that lack a test compound. A stock solution of trimethoprim is prepared to a concentration of 10 mg/ml in DMSO and stored at -20°C. A working solution of trimethoprim is prepared by diluting the trimethoprim stock solution to 10 µg/ml with 50 mM HEPES (pH 7.4). A 5 µl aliquot of the 10 µg/ml of trimethoprim working solution is added to the plate to provide a control. The bacteria and test compounds
15 (or trimethoprim control) are then incubated at 30°C for approximately 5 hours. If desired, the length and/or temperature of incubation can be increased or decreased. After incubating the cells and test compounds, the plates can be stored at -80°C if they are not going to be analyzed immediately. Before analysis, the plates should be returned to room temperature, e.g., by leaving the plates in a single layer for one hour at room temperature.

Assay for Induction of Promoter Activity: To assay for an increase in the activity of
20 the *panB* promoter, an increase in expression of the *lacZ* reporter gene expression (or expression of an alternative reporter gene) is measured. A 50 µl aliquot of 2X substrate buffer is added to each well of the plate. To produce a 10 ml sample of 2X substrate buffer, the following components are mixed and kept at room temperature: 0.4 ml of Galacton-
25 Star™ substrate (3-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(4'chloro)-tricyclo-[3.3.1.1^{3,7}] decan}-4-yl)phenyl-B-D-galactopyranoside; Tropix, Inc., Cat.# GS100); 2.0 ml of Sapphire II™ luminescence signal enhancer (Tropix, Inc., Cat.#LAX250); and 7.6 ml of lysis buffer (0.026% Na Deoxycholic acid, 0.053% CTAB, 265 mM NaCl, 395 mM HEPES, pH 7.5). The 2X substrate buffer is stable for approximately 2 hours. The plates are
30 incubated at room temperature for 120 minutes before measuring β-galactosidase activity.

A chemiluminescent signal is produced by reaction of β -galactosidase with the Galacton-StarTM substrate (3-chloro-5-(4-methoxyspiro{1,2-dioxetane-3,2'-(4'chloro)-tricyclo-[3.3.1.1^{3,7}] decan}-4-yl)phenyl-B-D-galactopyranoside). Chemiluminescence is measured using a TopCountTM microplate reader set as follows: data mode = seconds per count; count time = 0.1 minute; count delay = 0.10 minute; background subtract = none; half-life correction = no; sample screening = no; and quench indicator = tSIS. Generally, chemiluminescence should be measured before significant signal decay occurs. The read window for the substrate, during which time the plates should be analyzed, is approximately 120 minutes to 180 minutes after initiating the reaction.

The mean and standard deviation of the results obtained for the reactions on a particular plate are measured (excepting the trimethoprim control wells). A chemiluminescent signal that is at least 3 times the standard deviation, plus the mean, indicates that the test compound is a lead or candidate compound, i.e., a compound that increases the activity of the promoter and is a tetrahydrofolate biosynthesis inhibitor. The trimethoprim controls described above should produce a chemiluminescent signal that is at least equal to 4 times the mean. The general quality of the analysis of the plate can be determined using Spotfire Pro version 4.0 data analysis software (Spotfire, Inc.; Cambridge, MA) by assessing parameters such as the overall hit rate, the random nature of the position of hits, and signal strength consistency over all plates and analyses. Compounds that inhibit tetrahydrofolate biosynthesis may or may not be bacteriocidal or bacteriostatic. Nevertheless, such compounds can be used in the development of antibacterial drugs through the use of standard medicinal chemistry techniques. Thus, the invention includes methods for preparing an antibacterial agent by: screening multiple test compounds as described herein to identify candidate compounds that upregulate promoter activity and therefore are tetrahydrofolate biosynthesis inhibitors; isolating one or more lead compounds from the candidate compounds; determining whether the compound inhibits the growth of a bacterium, in which case the compound can be formulated as an antibacterial agent; and, optionally, derivatizing the lead compound(s) through medicinal chemistry to produce a derivative(s) that inhibits the growth of a bacterium; and formulating the derivative as an antibacterial agent. Optionally, the compound can be characterized in a biochemical assay to determine which step it inhibits in the tetrahydrofolate biosynthesis pathway and/or confirm that the protein is a tetrahydrofolate biosynthesis inhibitor.

Example 3: Confirmatory Metabolite Uptake Assay

Test compounds that cause an induction of promoter activity of at least 3 times the standard deviation, plus the mean, can be further tested (e.g., at 10 µg/ml and 50 µg/ml) in an optional, “confirmatory” assay. In one implementation, a cell-based metabolite uptake assay is used as the “confirmatory” assay. Cells are grown in Luria Broth supplemented with 100 µg/ml thymidine. Carboxyl ¹⁴C labeled PABA (60 mCi/mmol) is stored in ethanol at a concentration of 5·10⁶ cpm/ml at 4°C. After approximately 16 hours of growth, cells are diluted 100 fold and ¹⁴C labeled PABA is added to the media at about 6700 cpm/ml. Test compounds are also added to the media of test cultures. Additional cultures are prepared with no additive or with trimethoprim or sulfamethoxazole added as controls. The cultures are grown with shaking for 20 to 24 hours at 30°C. Subsequently, cells are filtered through a Sartorius 0.22 µm cellulose nitrate filter and washed with media. Samples are processed in parallel on a manifold. The filters are then placed in scintillation fluid and counted using a scintillation counter. Cell densities can be determined by measuring the optical density of the remainder of the culture. The amount of PABA incorporated can be determined by dividing the scintillation counts detected by the optical density. The mean and standard deviation are determined using the values obtained with multiple wells (minus the values of trimethoprim controls). An example of the effect of trimethoprim and sulfathiazole on PABA uptake is illustrated in Fig. 1 (Herrington. *Ibid.*).

Example 4: Biochemical Assay of Inhibition of Tetrahydrofolate Biosynthesis:

If desired, test compounds identified as candidate antibacterial agents in the above-described assays can be further tested for their ability to inhibit tetrahydrofolate biosynthesis in a biochemical assay. For example, compounds identified in the above-described assay can be further tested for their ability to inhibit the enzymes encoded by tetrahydrofolate biosynthesis genes, such as *mtrA*, *folA*, *folK*, *sul*, *pabA*, *pabB*, *pabC*, *folC*, and *dfrA*. The reactions catalyzed by each of these gene products are set forth above in Table 1, and methods for assaying these reactions are known in the art.

In one implementation, candidate compounds can be assayed for their ability to inhibit *B. subtilis* dihydrofolate reductase as described in Myoda *et al.* (1984) *Gene* 29:135-143. *B. subtilis* cells resuspended in 50 mM Tris·HCl, pH 7.4 10 mM 2-mercaptoethanol, and incubated with 200 µg/ml lysozyme and 10 µg/ml deoxyribonuclease I for 30 minutes at

37°C. Cells are then sonicated at 4°C with three 90 second bursts interspersed with 30 second cooling intervals. Cell debris is pelleted by centrifugation at 30 000 g for 20 minutes. Protein concentration is determined by the Bradford method using a kit (Bio-Rad). A 1 ml reaction mixture is supplemented with 50 nmol NADPH, 10 µmol 2-mercaptoethanol, and 25 mM potassium phosphate pH 7.0. The reaction is carried out at 30°C and followed with a spectrophotometer at 340 nm. The candidate compound is added at a range of concentrations to one set of reaction mixtures while another set of reaction mixtures lacking the candidate compound is tracked in parallel as a control. The reaction can be initiated by the addition of 60 nmol dihydrofolic acid (DHF). Dihydrofolate reductase specific activity can expressed as nmol of DHF reduced/min/mg of protein using an extinction coefficient of 11 650.

Other Embodiments

It is to be understood that, while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

- 1 1. A method for determining whether a test compound is an inhibitor of bacterial
2 tetrahydrofolate biosynthesis, the method comprising:
3 (i) contacting a bacterial cell with a test compound, wherein the bacterial cell
4 contains a promoter, the activity of which is increased in the presence of a compound that
5 inhibits tetrahydrofolate biosynthesis; and
6 (ii) measuring activity of the promoter, wherein an increase in activity, relative to
7 the level of activity of the promoter in the absence of the test compound, indicates that the
8 test compound is an inhibitor of bacterial tetrahydrofolate biosynthesis.

- 1 2. A method of claim 1, wherein the promoter is *panB*.

- 1 3. A method of claim 2, wherein the *panB* promoter comprises a nucleic acid
2 sequence as set forth in SEQ ID NO:1 or a fragment thereof.

- 1 4. A method of claim 1, wherein the activity of the promoter is measured by an
2 antibody specific for a polypeptide selected from the group consisting of: aspartate 1-
3 decarboxylase, pantothenate synthase, and ketopantoate hydroxymethyltransferase.

- 1 5. A method of claim 1, wherein the activity of the promoter is measured by an
2 assay for the activity of an enzyme selected from the group consisting of: aspartate 1-
3 decarboxylase, pantothenate synthase, and ketopantoate hydroxymethyltransferase.

- 1 6. A method of claim 1, wherein the activity of the promoter is measured by
2 detecting the RNA species transcribed from the gene regulated by the promoter.

- 1 7. A method of claim 1, wherein the cell contains the promoter operably linked to a
2 reporter gene.

1 8. A method of claim 7, wherein the reporter gene is selected from the group
2 consisting of *lacZ*, *cat*, *gus*, a luciferase gene, and a green fluorescent protein gene.

1 9. A method for determining whether a test compound is an inhibitor of bacterial
2 tetrahydrofolate biosynthesis, the method comprising:

3 (i) contacting a bacterial cell with a test compound, wherein the bacterial cell
4 contains

5 (a) a promoter, the activity of which is increased in the presence of a compound
6 that inhibits tetrahydrofolate biosynthesis, operably linked to

7 (b) a reporter gene; and

8 (ii) measuring activity of the promoter, wherein an increase in activity, relative to the
9 level of activity of the promoter in the absence of the test compound, indicates that the test
10 compound is an inhibitor of bacterial tetrahydrofolate biosynthesis.

1 10. A method of claim 9, wherein the reporter gene is selected from the group
2 consisting of *lacZ*, *cat*, *gus*, a luciferase gene, and a green fluorescent protein gene.

1 11. A method of claim 9, wherein measuring an increase in activity of the promoter
2 comprises measuring binding of antibodies to a product of the reporter gene.

1 12. A method of claim 9, wherein the promoter is *panB*.

1 13. A method of claim 12, wherein the *panB* promoter comprises a nucleic acid
2 sequence as set forth in SEQ ID NO:1 or a fragment thereof.

1 14. A method for determining whether a test compound is an antibacterial agent, the
2 method comprising:

3 (i) contacting a bacterial cell with a test compound, wherein the bacterial cell
4 contains

5 (a) a promoter, the activity of which is increased in the presence of a compound
6 that inhibits tetrahydrofolate biosynthesis, operably linked to

7 (b) a reporter gene;

8 (ii) measuring activity of the promoter, wherein an increase in activity, relative to the
9 level of activity of the promoter in the absence of the test compound, indicates that the test
10 compound is an inhibitor of bacterial tetrahydrofolate biosynthesis; and

11 (iii) determining whether the inhibitor of tetrahydrofolate biosynthesis inhibits the
12 growth of a bacterium, wherein a compound that inhibits the growth of a bacterium is an
13 antibacterial agent.

1 15. A method of claim 14, further comprising assaying the test compound for its
2 ability to inhibit tetrahydrofolate biosynthesis.

1 16. A method of claim 15, wherein inhibition of tetrahydrofolate biosynthesis is
2 detected as inhibition of *para*-aminobenzoic acid (PABA) uptake into cells.

1 17. A method of claim 15, wherein inhibition is measured in a biochemical assay with
2 a cell extract for an enzyme activity which is required for tetrahydrofolate biosynthesis.

3
4 18. A method of claim 17 wherein the enzyme activity assayed is selected from the
5 group consisting of: GTP cyclohydrazase, 7,8 dihydroneopterin aldolase, 6-hydroxymethyl-
6 7,8-dihydropterin pyrophosphokinase, dihydropteroate synthase, aminodeoxychorismate
7 synthase, aminodeoxychorismate lyase, dihydrofolate:folyl-polyglutamate synthase, and
8 dihydrofolate reductase.

1 19. A method of claim 14, wherein the promoter is *panB*.

1 20. A method for treating a bacterial infection in an organism, the method comprising
2 administering to the organism an antibacterial agent identified by the method of claim 14, in
3 an amount effective to treat the bacterial infection.

1 21. A method of claim 20, wherein the organism is a mammal.

1 22. A method of claim 21, wherein the mammal is a human.

1 23. A method of claim 20, wherein the bacterial infection is caused by *Streptococcus*
2 *pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus endocarditis*,
3 *Streptococcus faecium*, *Streptococcus sanguis*, *Streptococcus viridans*, or *Streptococcus*
4 *hemolyticus*.

1 24. A method of claim 20, wherein the bacterial infection is an infection by a
2 pathogenic bacterium.

1 25. A method of claim 20, wherein the bacterial infection is an infection by a non-
2 pathogenic bacterium.

1 26. A composition comprising an antibacterial agent identified by the method of
2 claim 14 and a pharmaceutically acceptable carrier.

1 27. A method of preparing an inhibitor of bacterial tetrahydrofolate biosynthesis, the
2 method comprising:
3 screening multiple test compounds by the method of claim 9;
4 identifying candidate compounds that increase promoter activity;
5 identifying, and selecting from the candidate compounds a lead compound that
6 inhibits bacterial tetrahydrofolate biosynthesis; and
7 formulating the selected lead compound as an inhibitor of bacterial tetrahydrofolate
8 biosynthesis.

1 28. A method of preparing an antibacterial agent, the method comprising:
2 screening multiple test compounds by the method of claim 14;
3 identifying candidate compounds that upregulate promoter activity;
4 identifying and selecting from the candidate compounds a lead compound that
5 inhibits growth of a bacterium; and
6 formulating the selected lead compound as an antibacterial agent.

1 29. A method for preparing an inhibitor of bacterial tetrahydrofolate biosynthesis, the
2 method comprising:
3 screening multiple test compounds by the method of claim 9;
4 identifying candidate compounds that increase promoter activity;
5 isolating a lead compound from the candidate compounds;
6 derivatizing the isolated lead compound, to produce derivatives of the lead
7 compound;
8 identifying a derivative that inhibits bacterial tetrahydrofolate biosynthesis; and
9 formulating the identified derivative as an inhibitor of bacterial tetrahydrofolate
10 biosynthesis.

1 30. An inhibitor of bacterial tetrahydrofolate biosynthesis prepared by the method of
2 claim 29.

1 31. A method for inhibiting bacterial tetrahydrofolate biosynthesis in bacteria
2 infecting an organism, the method comprising administering to the organism an antibacterial
3 agent identified by the method of claim 30, in an amount effective to inhibit bacterial
4 tetrahydrofolate biosynthesis in the bacteria.

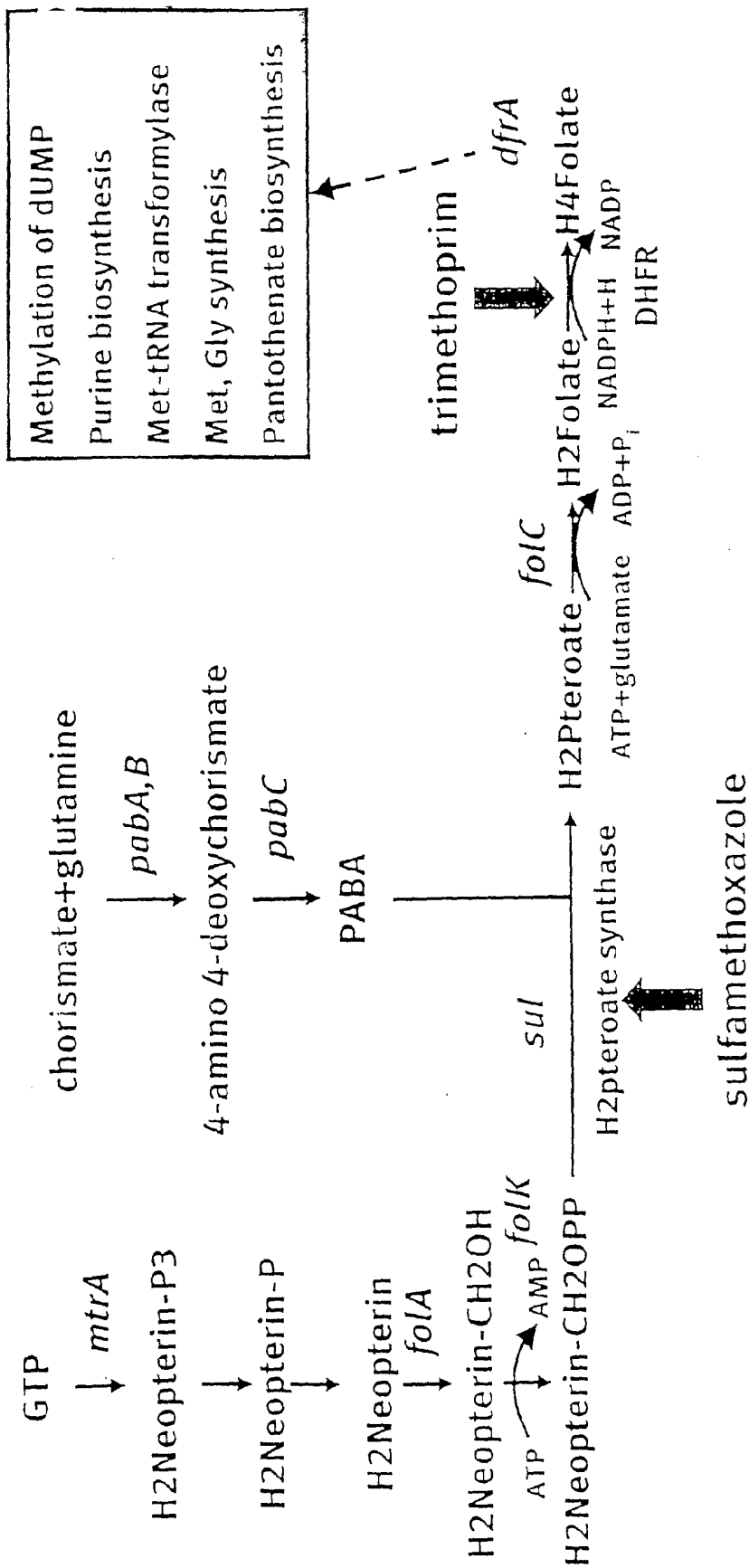
1 32. A method for preparing an antibacterial agent, the method comprising:
2 screening multiple test compounds by the method of claim 14;
3 identifying candidate compounds that increase promoter activity;
4 identifying and selecting a lead compound that inhibits growth of a bacterium
5 from the candidate compounds;
6 derivatizing the selected lead compound to produce derivatives of said lead
7 compound;
8 identifying a derivative that inhibits growth of a bacterium; and
9 formulating the identified derivative as an antibacterial agent.

1 33. An antibacterial agent prepared by the method of claim 32.

1 34. A composition comprising an antibacterial agent of claim 33 and a
2 pharmaceutically acceptable carrier.

1 35. A method for inhibiting growth of bacteria in an organism having a bacterial
2 infection, the method comprising administering to the organism a composition of claim 34, in
3 an amount effective to inhibit growth of bacteria in the organism

Folate biosynthesis in *B. subtilis*



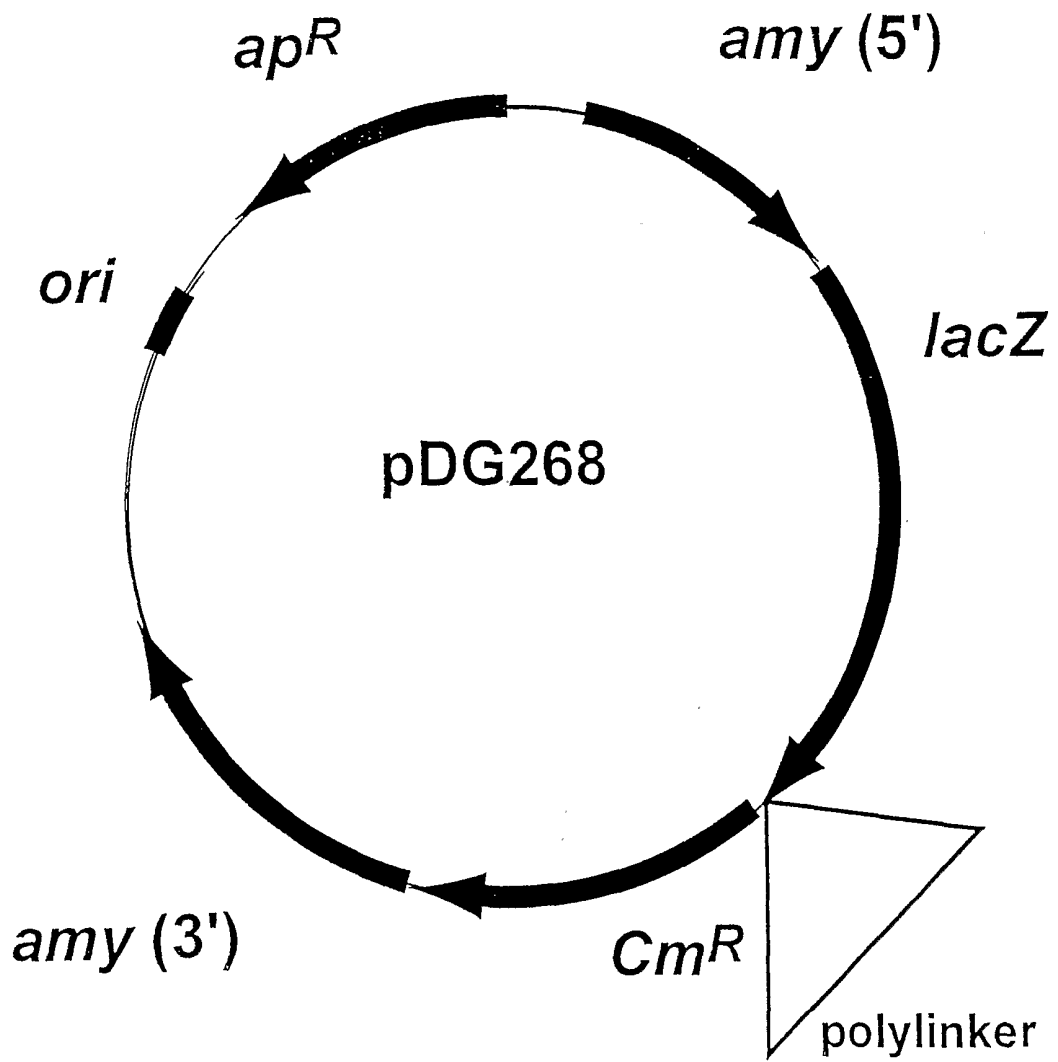


FIG. 2

lacZ-assay of pan. Fusion strain
treated with trimethoprim 5 hr.



FIG. 3

lacZ-assay of pan. Fusion strain
treated with sulfamethoxazole 5 hr.



FIG. 4