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(72) LEVY, STUART B., US
(71) TRUSTEES OF TUFTS COLLEGE, US

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(54) **METHODE ET COMPOSITIONS PERMETTANT DE REDUIRE LA TOLERANCE BACTERIENNE DE DESINFECTANTS ET DE SOLVANTS ORGANIQUES**

(54) **METHODS AND COMPOSITIONS FOR REDUCING BACTERIAL TOLERANCE OF DISINFECTANTS AND ORGANIC SOLVENTS**

(57) L'invention concerne des méthodes et des compositions permettant de manipuler la résistance bactérienne de compositions antibactériennes non antibiotiques, de désinfectants et de solvants organiques; des méthodes permettant de rendre des cellules bactériennes sensibles à des compositions antibactériennes non antibiotiques; des méthodes permettant de diminuer la sélection de mutants bactériens présentant un phénotype de multirésistance aux antibiotiques à l'aide de compositions antibactériennes non antibiotiques; des méthodes permettant de tester la capacité de compositions antibactériennes non antibiotiques à sélectionner ou induire un phénotype de multirésistance aux antibiotiques dans les bactéries; des méthodes permettant d'augmenter ou de réduire la tolérance bactérienne des solvants organiques en augmentant ou réduisant l'activité des pompes d'écoulement des solvants organiques bactériens. Cette invention décrit également des compositions utiles pour les méthodes précitées.

(57) The invention relates to methods and compositions for manipulating bacterial resistance to non-antibiotic antibacterial compositions, disinfectants and organic solvents. The invention provides methods for rendering bacterial cells susceptible to non-antibiotic antibacterial compositions. Also provided are methods to reduce the selection of bacterial mutants having a multiple antibiotic resistance phenotype by non-antibiotic antibacterial compositions. The invention also provides methods for testing the ability of non-antibiotic antibacterial compositions to select for or induce a multiple antibiotic resistance phenotype in bacteria. Also provided are methods for increasing or decreasing bacterial tolerance to organic solvents by increasing or decreasing the activity of bacterial organic solvent efflux pumps. Compositions useful in the foregoing methods are also provided.

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(71) Applicant: TRUSTEES OF TUFTS COLLEGE [US/US]; 4th floor, Ballou Hall, Medford, MA 02155 (US).

(72) Inventor: LEVY, Stuart, B.; 144 Warren Avenue, Boston, MA 02116 (US).

(74) Agent: WILLIAMS, Megan, E.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR REDUCING BACTERIAL TOLERANCE OF DISINFECTANTS AND ORGANIC SOLVENTS

(57) Abstract

The invention relates to methods and compositions for manipulating bacterial resistance to non–antibiotic antibacterial compositions, disinfectants and organic solvents. The invention provides methods for rendering bacterial cells susceptible to non–antibiotic antibacterial compositions. Also provided are methods to reduce the selection of bacterial mutants having a multiple antibiotic resistance phenotype by non–antibiotic antibacterial compositions. The invention also provides methods for testing the ability of non–antibiotic antibacterial compositions to select for or induce a multiple antibiotic resistance phenotype in bacteria. Also provided are methods for increasing or decreasing bacterial tolerance to organic solvents by increasing or decreasing the activity of bacterial organic solvent efflux pumps. Compositions useful in the foregoing methods are also provided.

METHODS AND COMPOSITIONS FOR REDUCING BACTERIAL TOLERANCE OF DISINFECTANTS AND ORGANIC SOLVENTS

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Field of the Invention

This invention relates to methods and compositions for manipulating bacterial resistance to non-antibiotic antibacterial compositions, disinfectants and organic solvents.

Background of the Invention

Antibiotic or antimicrobial substances have long been used to inhibit the growth of bacteria or other microbes and to treat bacterial or microbial infections in humans, other animals, and in tissue culture. The use of antibiotics or antimicrobials in a treatment regimen, however, has the undesirable effect of selecting for bacteria or other microbes which are resistant to those antibiotics or antimicrobials which are administered or applied. As a result, treatment regimens can be adversely affected or, in some cases, rendered ineffective. This necessitates a continual search for new antibiotics and antimicrobials.

Of particular interest is the discovery of bacteria which express a multiple antibiotic resistance phenotype (Mar). This phenotype entails simultaneous resistance to a multiplicity of antibiotics which are unrelated in chemical structure. The appearance of such bacteria and infections by such bacteria greatly increase the difficulty of identifying effective antibiotics and treating infections in humans or other animals.

Multiple antibiotic resistance in bacteria is most commonly associated with the presence of plasmids and/or transposons which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype. Multiple antibiotic resistance associated with the chromosome, however, has been reported in *Klebsiella, Enterobacter, Serratia* (Gutmann et al., *J. Infect. Dis.* 151:501-507, 1985), *Neisseria* (Johnson and Morse, *Sex. Transm. Dis.* 15:217-224, *1988),* and *Escherichia* (George and Levy, *J. Bacteriol.* 155: 531-540, 1983).

Bacteria expressing a chromosomal multiple antibiotic resistance phenotype can be isolated by selecting bacteria with a single antibiotic and then screening for cross-resistance to structurally unrelated antibiotics. For example, George and Levy initially described a chromosomal multiple antibiotic resistance system which exists in Escherichia coli and which can be selected by a single drug, e.g., tetracycline or chloramphenicol (George and Levy, 1983). In addition to resistance to the selective agents, the Mar phenotype includes resistance to structurally unrelated agents, including nalidixic acid, rifampin, penicillins, and cephalosporins (George and Levy 1983) as well as fluoroquinolones (Cohen et al. 1989).

The chromosomal gene locus which correlates with the Mar phenotype observed in *E. coli* has been identified. The chromosomal *mar* locus, located at 34 min on the *E. coli* chromosomal map, is involved in the regulation of intrinsic susceptibility to structurally unrelated antibiotics (Cohen et al., *J. Bacteriol.* 175: 1484-1492, 1993; Cohen et al., *Antimicrob. Agents and Chemother.* 33:1318-1325, 1989; Cohen et al., *J. Bacteriol.* 170:5416-22, 1988; Goldman et al., *Antimicrob. Agents Chemother.* 40: 1266-1269, 1996), as well as the expression of antioxidant genes (Ariza et al., *J. Bacteriol.* 176: 143-148, 1994; Greenberg et al., *J. Bacteriol.* 173 :4433-4439, 1991) and internal pH homeostasis (Rosner and Slonczewski, *J. Bacteriol.* 170:5416-22, 1994). The *mar* locus consists of two transcription units *(marC* and *marRAB)* which are divergently transcribed from a central putative operator-promoter region *(marO)* (Cohen et al., 1993; Goldman et al., 1996). *marR* is the repressor of the *marRAB* operon (Cohen et al., 1993; Martin and Rosner, *Proc. Natl. Acad. Sci. USA* 92:5465-5460, 1995; Seoane and Levy, *J. Bacteriol.* 177:3414-3419, 1995). Mutations in *marR* result in increased expression of the *marRAB* operon. Overexpression of *marA* alone is sufficient to produce the multiple antibiotic resistance phenotype (Cohen et al., 1993; Gambino et al., *J. Bacteriol.* 175:2888-2894, 1993; Yan et al., Abstr. A-26, p. 5, *In* Abstracts of the 1992 General Meeting of the American Society for Microbiology, American Society for Microbiology, Washington, DC, 1992). *marB* has no effect of its own; however, when it is present on the same construct with *marA*, it produces a small increase in antibiotic resistance (White et al., Abst A-104, p. 20. *In* Abstracts of the 1994 General Meeting of the American Society for Microbiology, American Society for Microbiology, Washington, DC 1994). The function of *marC* is unknown; however, it also appears to enhance the multiple antibiotic resistance phenotype when cloned on the same DNA fragment with the *marRAB* operon (Goldman et al., 1996; White et al., 1994).

Overexpression of *marA* confers multiple antibiotic resistance via increased efflux of antibiotics, including fluoroquinolones, tetracycline, and chloramphenicol (Cohen et al., 1989; George and Levy, 1983; McMurry et al., *Antimicrob. Agents Chemother.* 38:542-546, 1994). Transcription of the *acrAB* operon,

which encodes a multi-drug efflux pump whose expression is modulated by global stress signals (Ma et al., *Mol. Microbiol.* 16:45-55, 1995; Ma et al., *Mol. Microbiol.* 19:101-112, 1996), was shown to be elevated in strains containing *marR* mutations and displaying the Mar phenotype (Okusu et al., *J. Bacteriol.* 178:306-308, 1996). Moreover inactivation of *acrAB* led to increased antibiotic susceptibility in wild type and Mar mutants (Okusu et al., 1996).

More recently, mutations of *marR* have been found in clinical isolates resistant to quinolones (Maneewannakul and Levy, 1996). Thus *mar* mutants can be selected under clinical conditions and not merely under controlled laboratory conditions. Early *mar* mutants (i.e., "first-step" *mar* mutants) remain susceptible to many common antibiotics, although such mutants can achieve levels of clinical resistance to certain antibiotics, including tetracycline, nalidixic acid and rifampin (reviewed by Alekshun and Levy, *Antimicrob. Agents Chemother.* 1997). First-step *mar* mutants thus may serve as precursors of bacterial mutants which display higher levels of resistance resulting from additional mutations on the chromosome. Thus it has been demonstrated that antibiotics can select for mutations in chromosomal gene loci which confer multiple antibiotic resistance under clinical conditions.

Non-antibiotic antibacterial compositions such as disinfectants are widely used in both clinical and consumer environments for reducing bacterial contamination of work surfaces, equipment, products and the like. These non-antibiotic antibacterial compositions have been incorporated into a wide spectrum of cleansers, disinfectant compositions, soaps, lotions, plastics, etc. It is not known whether exposure of bacteria to non-antibiotic antibacterial compositions also can select for bacterial mutants, including those which display a multiple antibiotic resistance phenotype.

Summary of the Invention

It has now been discovered that bacterial mutants having multiple antibiotic resistance can be selected by non-antibiotic antibacterial agents such as common disinfectants. It further has been discovered that the phenotype of the multiple antibiotic resistant mutants selected by a non-antibiotic antibacterial agent results from mutations in chromosomal gene loci which regulate expression of efflux pumps, which loci have been implicated in multiple antibiotic resistance phenotypes as described above. The efflux pumps actively pump out the non-antibiotic antibacterial agents, as well as organic solvents and antibiotics, thereby rendering the mutant bacteria resistant to all of the foregoing compounds.

According to one aspect of the invention, a method is provided for inhibiting the selection and/or propagation of a bacterial mutant that overexpresses an efflux pump. Bacteria are contacted with an agent that binds to a gene locus (the

expression of the gene locus enhances expression of the efflux pump) or an expression product thereof. in an amount effective to inhibit the gene locus-enhanced expression of the efflux pump. In preferred embodiments. the gene locus is selected from the group consisting of a *mar* locus. a *sox* locus and a *rob* locus. Also in preferred embodiments. the efflux pump is acr-like. including the *acrAB* efflux pump.

The agent can be selected from the group consisting of chemicals, antisense nucleic acids. antibodies. ribozymes. and proteins which repress expression of the gene locus. A preferred embodiment is an agent that is an antisense nucleic acid, and in particularly preferred embodiments. the agent is antisense to the *mar* locus, *sox* locus and/or *rob* locus. Another preferred embodiment is chemical inhibitors of efflux pumps, particularly L-phenylalanyl-L-arginyl-β-naphthylamide.

According to another aspect of the invention. a method is provided for rendering bacterial cells more susceptible to a non-antibiotic bactericidal or bacteriostatic agent that is a substrate of an efflux pump. An inhibitor of a gene locus or an expression product thereof is administered to a bacterial cell. wherein the expression of the gene locus enhances expression of an efflux pump. In preferred embodiments the gene locus is selected from the group consisting of a *mar* locus. a *sox* locus and a *rob* locus. In other preferred embodiments the efflux pump is *acr*-like and can be *acrAB*. The preferred inhibitors are as described above.

According to still another aspect of the invention, a method is provided for rendering bacterial cells more susceptible to a non-antibiotic bactericidal or bacteriostatic agent that is a substrate of an efflux pump. The method involves administering to the bacterial cell an inhibitor of the efflux pump. In preferred embodiments the efflux pump is *acr*-like and can be *acrAB*. Preferably the inhibitor is selected from the group consisting of about 4% ethanol. methanol. hexane. minocycline and L-phenylalanyl-L-arginyl-β-naphthylamide.

According to another aspect of the invention. a method is provided for modulating (increasing or decreasing) the ability of bacterial cells to survive in an organic solvent. In certain embodiments the method involves enhancing expression in the bacterial cells of an organic solvent bacterial efflux pump by growing the bacterial cells in the presence of a non-*mar/sox/rob* inducing agent, wherein the agent induces the overexpression of the organic solvent bacterial efflux pump. The agent can be a gene encoding an *acr*-like pump, the *acrAB* pump, or expression products thereof. In other embodiments the method involves reducing expression in the bacterial cells of an organic solvent bacterial efflux pump by growing the bacterial cells in the presence of an agent, wherein the agent reduces the expression of the organic solvent bacterial efflux pump. The agent can be an antisense nucleic acid which binds to a gene locus encoding an *acr*-like pump, especially the *acrAB* pump, a gene locus which enhances expression

of an efflux pump, such as *marA*, *soxA* and *robA*, and the like. The agent also can be a ribozyme or a protein which represses expression of the gene locus. The agent also can be an antibody to an expression product of the foregoing genes. The agent also can be a chemical compound which reduces expression of the efflux pump, or reduces activity of the efflux pump, such as L-phenylalanyl-L-arginyl-β-naphthylamide.

According to another aspect of the invention, a method is provided for testing the ability of a non-antibiotic composition to induce a multiple antibiotic resistance phenotype in a bacterium. The bacterium is contacted with the non-antibiotic composition. The expression of a bacterial gene locus is determined, the altered expression of which is indicative of induction of the multiple antibiotic resistance phenotype in the bacterium. Then, the result of this determination is compared with a control, wherein altered expression of the bacterial gene locus indicates that the non-antibiotic composition induces the multiple antibiotic resistance phenotype in the bacterium. In preferred embodiments, the gene locus is selected from the group consisting of a *mar* locus, a *sox* locus, a *rob* locus and an *acr*-like efflux pump locus. In one particular embodiment the efflux pump locus is *acrAB*. The foregoing method can be carried out using a non-antibiotic composition that is an inactive ingredient. The inactive ingredient can be a non-bactericidal ingredient. The inactive ingredient also can be a non-bacteriostatic ingredient.

According to another aspect of the invention, a composition is provided. The composition includes a non-antibiotic bactericidal or bacteriostatic first agent and a second agent that inhibits the expression of activity of an efflux pump. In one embodiment, the second agent inhibits the expression of a gene locus or an expression product thereof, wherein the expression of the gene locus enhances expression of the efflux pump. In preferred embodiments, the second agent is selected from the group consisting of antisense nucleic acids, antibodies, ribozymes and proteins that repress expression of the gene locus. In one preferred embodiment the second agent inhibits an *acr*-like efflux pump, and particularly preferred is an antisense nucleic acid. The second agent also can be selected from the group consisting of 4% ethanol, methanol, hexane, minocycline and L-phenylalanyl-L-arginyl-β-naphthylamide. The preferred second agent is [preferred inhibitor]. The first agent in some embodiments is selected from the group consisting of triclosan, pine oil, quaternary amine compounds including alkyl dimethyl benzyl ammonium chloride, chloroxylenol, triclocarbon, disinfectants and organic solvents. These and other aspects of the invention are described in greater detail below.

Brief Description of the Figures

Figure 1 shows the Northern blot analysis of *marRAB* mRNA in bacterial mutants.

Detailed Description of the Invention

The invention is based on the discovery that non-antibiotic antibacterial compositions and organic solvents select for mutant bacteria which are resistant not only to the non-antibiotic antibacterial compositions, but also to a range of antibiotics (i.e. a multiple antibiotic resistant phenotype) and also to organic solvents. All of the foregoing compounds are pumped out of bacteria by efflux pumps, i.e., the foregoing compounds are substrates for the efflux pumps. Based on these discoveries it is now possible to enhance the antibacterial properties of non-antibiotic antibacterial compositions and also reduce the selection of bacterial mutants having a multiple antibiotic resistance phenotype by such compositions. The invention also provides methods for testing the ability of non-antibiotic antibacterial compositions to select for or induce a multiple antibiotic resistance phenotype in bacteria. The invention also provides methods for increasing or decreasing bacterial tolerance to organic solvents by increasing or decreasing the activity of bacterial organic solvent efflux pumps, such as by increasing or decreasing expression of an efflux pump, increasing or decreasing expression of genes which positively regulate efflux pump gene loci, and the like. Compositions useful in the foregoing methods are also provided.

As used herein, a non-antibiotic antibacterial composition is a molecule or combination of molecules which are bactericidal or bacteriostatic, but which are not antibiotics. "Antibiotics" are those bactericidal or bacteriostatic compounds which are administered *in vivo* to people, animals or plants which have a bacterial infection, or which are used *in vitro* for research on bacterial infections of animals. A non-antibiotic antibacterial composition is not administered to a subject, but rather is used as a disinfectant for killing bacteria or reducing the growth rate of a population of bacteria. Non-antibiotic antibacterial compositions are added as the active ingredients in a variety of industrial and household disinfectants, such as LYSOL™, PINE-SOL™, and the like. Non-antibiotic antibacterial compositions also are added as the antibacterial active ingredient in non-disinfectant compositions such as soaps, lotions, cleansers and the like. More recently, non-antibiotic antibacterial composition have been incorporated into plastics for making a variety of articles of manufacture which have resistance to bacterial growth.

The non-antibiotic antibacterial compositions, as used herein, may have active and inactive ingredients. The active ingredients are, of course, the bactericidal or bacteriostatic agents which have the effect of slowing or stopping growth of populations of bacteria, or even killing such populations of bacteria. Active bactericidal or bacteriostatic ingredients include triclosan, pine oil, quaternary amine compounds such as alkyl dimethyl benzyl ammonium chloride, chloroxylenol, triclocarbon, and other

well known disinfectants. The inactive ingredients are the balance of the components of the non-antibiotic antibacterial compositions, including surfactants and other cleansing agents, binders, bulking agents and other compounds. Thus non-antibiotic antibacterial compositions refers both to the active ingredient of the compositions as well as the compositions themselves.

The invention provides methods for inhibiting the selection or propagation of a bacterial mutant that overexpresses an efflux pump. By "inhibiting the selection or propagation", it is meant that the method provides inhibition of selection of a multiple antibiotic resistant bacterial mutant (i.e., the initial mutation event which causes the induction of an efflux pump) and/or inhibition of propagation of a multiple antibiotic resistant bacterial mutant (i.e., growth and/or replication of such bacteria).

The invention also provides methods for rendering bacterial cells more susceptible to non-antibiotic antibacterial compositions by administering to the bacterial cells inhibitors of an efflux pump or a gene locus which enhances expression of the efflux pump, or an expression product thereof. By "administered to", it is meant that the bacterial cells are contacted with the inhibitor for a time sufficient to permit inhibition of the efflux pump or gene locus.

The invention further provides methods for increasing or decreasing organic solvent tolerance of bacterial cells. In these methods, overexpression of an organic solvent efflux pump is induced or decreased by growing the cells in the presence of an agent. By induced "overexpression" it is meant that the organic solvent efflux pump is expressed at a higher level in bacterial cells grown in the presence of an inducing agent than in identical bacterial cells grown under identical conditions but without the agent, i.e., a level of expression that is sufficient to increase organic solvent tolerance. By decreased "overexpression" it is meant that the organic solvent efflux pump is expressed at a lower level in bacterial cells grown in the presence of an inhibiting agent than in identical bacterial cells grown under identical conditions but without the agent, i.e., a level of expression that is sufficient to reduce tolerance or increase organic solvent susceptibility. These methods can also confer organic solvent tolerance or susceptibility by modulating the activity of an efflux pump as described herein. Organic solvent tolerance or susceptibility can be determined by standard methodologies, including those exemplified in Example 2 below.

One of the features of antibacterial products is the reduction in bacterial populations in those products or on those products, or on surfaces to which such products are applied. As disclosed herein, non-antibiotic antibacterial products also can select for multiple antibiotic resistant bacteria. It would be useful to be able to determine which non-antibiotic antibacterial compositions select for deleterious mutants. Having determined that non-antibiotic antibacterial compositions can select for mutants, it is

also possible that other non-antibiotic compositions can select for mutations. Therefore the invention embraces methods for testing the ability of non-antibiotic compositions to induce a multiple antibiotic resistance phenotype. These methods permit testing of any non-antibiotic composition, including the inactive ingredients in cleansers, soaps, disinfectants and the like. In these methods, a bacterial culture is contacted with a non-antibiotic composition and the expression of a gene locus which is indicative of a multiple antibiotic resistant phenotype is determined. The gene locus expression can be determined by any convenient method, of which many are known in the art. These methods include enzyme assays comprising fusions of regulatory loci to a marker gene (e.g. as described for a *mar* regulatory locus in PCT published application W094/05810), amplification of gene transcripts (such as using polymerase chain reaction), hybridization methods including Northern blots, and measurement of protein expression including Western blots, ELISA, etc. The level of expression of the gene locus is then compared with a control to determine if the non-antibiotic compositions induced the multiple antibiotic resistant phenotype.

According to the invention, various agents which inhibit the expression or activity of an efflux pump or gene loci which control expression of the efflux pump are useful for reducing selection and/or propagation of mutant bacteria, and also render the cells more susceptible to non-antibiotic antibacterial compositions. These inhibitors are contacted with or administered to the bacterial cells to prevent the undesirable effects of the non-antibiotic antibacterial compositions. One convenient way to ensure contact of the appropriate bacterial cell populations is to include the inhibitors and agents in the non-antibiotic antibacterial compositions. Thus the invention further provides compositions comprising a non-antibiotic bactericidal or bacteriostatic first agent and a second agent which inhibits the expression or activity of an efflux pump, as described above. These compositions can be prepared according to the standard procedures used to prepare non-antibiotic antibacterial compositions. For example, a standard disinfectant composition such as PINE-SOL™ can have added to it an effective amount of an inhibitor of an efflux pump such as described in PCT published patent application W096/33285, or an antisense nucleic acid which binds to the efflux pump gene locus, etc.

By "effective amount" is meant an amount of the second agent which reduces the selection of mutants by the non-antibiotic first agent. Effective amounts can be determined using standard bacterial growth and mutation assays, including those provided herein. For example, various amounts of the second agent can be added to a non-antibiotic antibacterial composition, and the combined composition can be used as provided in the examples below to select bacterial mutants. Any amount of the second agent which reduces the number of mutants selected relative to the number of mutants

selected by the non-antibiotic antibacterial composition alone is an effective amount. One of ordinary skill in the art can determine with no more than routine experimentation what constitutes an effective amount of a second agent, and what amount of a second agent is optimal to prevent selection of mutants by the non-antibiotic antibacterial compositions. Effective amounts of other inhibitors and agents disclosed herein can be determined similarly.

As disclosed herein, inhibitors of the *marA* gene locus and other loci which regulate efflux pumps are effective to reduce the selection of antibiotic resistant bacterial mutants by non-antibiotic antibacterial compositions, and also potentiate the antibacterial properties of such compositions. The *marA* gene has been cloned and sequenced, the sequence deposited as GenBank accession number M96235. The *marA* gene has homologs in *E. coli*, as well as in other species of bacteria. Inhibitors of such *marA* homologs also are useful for reducing the selection of antibiotic resistant bacterial mutants and potentiating the antibacterial properties of non-antibiotic antibacterial compositions.

For example, the MarA protein is homologous to both SoxS, the effector of the *soxRS* regulon (Fawcett and Wolf, *Mol. Microbiol.* 14:669-679, 1994; Li and Demple, *J. Biol. Chem.* 269:18371 - 18377, 1994), and RobA, a small protein that binds to the *E. coli* replication origin and some stress gene promoters (Ariza et al., 1995; Cohen et al., 1995; Jair et al., *J. Bacteriol.* 178:2507-2513, 1996; Skarstad et al., J. *Biol. Chem.* 268:5365-5370, 1993). The *soxRS* regulon mediates the cell's response to oxidative stress (Amabile-Cuevas and Demple, *Nucleic Acids Res.* 19:4479-4484, 1991; Nunoshiba et al., J. Bacteriol. 174:6054-6060, 1992; Wu and Weiss, *J. Bacteriol.* 173:2864-2871, 1991). *sox S* genes include those found in *S. typhimurium* (GenBank accession number U61147) and *E. coli* (GenBank accession numbers X59593 and M60111). *robA* genes include those found in *E. coli* (GenBank accession numbers AE000509, U00096, M97495 and M94042).

Other known homologs of *marA* include those found in *Enterobacteriaceae* by nucleic acid hybridization under stringent conditions (Cohen et al., 1993). Other *marA* homologs include *pqrA*, identified in *Proteus vulgaris* (GenBank accession number D13561), *ramA* identified in *Klebsiella pneumonia* (GenBank accession number U19581), and *aarP* identified in *Providencia stuartil* (GenBank accession number L38718).

Additional homologs of *marA* (and other gene loci useful according to the invention) can be identified by conventional techniques. Such techniques include cloning by hybridization to *marA* or to known homologs thereof, and functional cloning. Cloning by hybridization involves subjecting *marA* or known homologs thereof to hybridization with nucleic acids of bacteria (preferably the chromosomal DNA) under

stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laborator Manual*, J. Sambrook, et al., eds., Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH_2PO_4(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 x SSC/0.1 x SDS at temperatures up to 65°C.

There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of homologs and alleles of nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

In general homologs typically will share at least 30% nucleotide identity and/or at least 40% amino acid identity to *mar/sox/rob* genes or to efflux pumps genes, or their polypeptide products respectively, in some instances will share at least 50% nucleotide identity and/or at least 65% amino acid identity and in still other instances will share at least 60% nucleotide identity and/or at least 75% amino acid identity. Watson-Crick complements of the foregoing nucleic acids also are embraced by the invention.

Functional cloning is useful to isolate homologs which do not share sufficient homology at the nucleotide or amino acid sequence level to permit cloning by nucleic acid hybridization, but which nevertheless are functional equivalents of the genes useful in the invention. Functional equivalents need not exhibit the same level of activity, merely activity of the same kind. For example, one phenotypic manifestation of *marA* expression is the induction of the expression of a set of genes, including *acrA*. A gene which induces substantially the same set of genes but at a lower level of expression would be considered a functional equivalent.

Functional cloning, as used herein, involves expression of a nucleic acid sequence in a bacterium and determining whether the expression of that sequence

confers a desired phenotype on the bacterium. It is known that *marA* homologs exhibit similar functional characteristics with respect to multiple antibiotic resistance phenotype. For example, overexpression of either *soxS* or *robA* in *E. coli* produces both increased organic solvent tolerance and low-level resistance to multiple antimicrobial agents (Ariza et al., *J. Bacteriol.* 177:1665-1661, 1995; Nakajima et al., *Biosci. Biotechnol. Biochem.* 59:1323-1325, 1995a; Nakajima et al., *Appl. Environ. Microbiol.* 61 :2302-2307, 1995b). Thus, for *marA* homologs, the desired phenotype can be multiple antibiotic resistance, induction of *mar*-regulated genes (see, e.g., U.S. Patent 5,650,321), and the like. For determining multiple antibiotic resistance, all that is necessary is to express the putative *marA* homolog in a non-multiple antibiotic resistant bacterium and determine whether the modified bacterium acquires resistance to more than one antibiotic, such as tetracycline, chloramphenicol, nalidixic acid, etc. *marA* homologs can be expressed according to standard procedures, such as transformation with an expression plasmid containing the marA homolog, introduction of one or more copies of the *marA* homolog on the bacterial chromosome via transposon-mediated insertion, etc.

The *acrAB* locus, positively regulated by *Mar*A (Ma et al., *Mol. Microbiol.* 16:45-55, 1995) and SoxS and RobA (Ma et al., *Mol. Microbiol.* 19:101-112, 1996), specifies a proton-motive-force-dependent multidrug efflux pump for a wide variety of mostly lipophilic substances (Ma et al., 1995; Nikaido, *Bacteriol.* 178:5853-5859, 1996; Nikaido, *Science* 264:382-387, 1994; Paulsen et al., *Microbiol. Rev.* 60:575-608, 1996). Mar mutants and wild type strains deleted of this locus become equally hypersusceptible to antibiotics (Okusu et al., *J. Bacteriol.* 178:306-308, 1996) suggesting that the *acrAB* pump confers an intrinsic resistance level which is then enhanced in Mar mutants.

The *acrA* and *acrB* genes have been cloned and sequenced. For example, the sequences of *acrAB* in *E. coli* are deposited as GenBank accession number U00734. The *acrAB* genes have homologs in *E. coli*, as well as in other species of bacteria. Sequence homologs of *acrAB* efflux pumps are referred to herein as *"acr*-like" efflux pumps. Isolation of *acr*-like efflux pumps and other efflux pumps can be carried out according to the methods described above for nucleic acid hybridization and functional cloning. Inhibitors of such *acrAB* homologs also are useful for reducing the selection of antibiotic resistant bacterial mutants and potentiating the antibacterial properties of non-antibiotic antibacterial compositions.

Agents which induce overexpression of *acr*-like efflux pumps are useful in promoting organic solvent tolerance. Inducers of efflux pumps include genes which encode the various efflux pumps which when expressed in a bacterium as a nucleic acid operably linked to a promoter can increase the numbers of efflux pump protein molecules in the bacterium. Agents also include molecules which inhibit the function of

efflux pump regulatory genes. For example, antisense nucleic acids which bind to *acrR* and prevent its transcription or translation would function as inducers of *acrAB*. Efflux pumps can also be induced by mutation of regulatory genes (such as *acrR* for the *acrAB* pump).

Agents useful in decreasing the expression or activity of an efflux pump for increasing organic solvent susceptibility (decreasing organic solvent tolerance) are provided in the following paragraphs.

Agents which bind to a gene locus which mediates enhanced expression of an efflux pump (such as the *mar/sox/rob* class of genes) or a nucleic acid expression product thereof include antisense nucleic acids, ribozymes and regulatory proteins such as repressor proteins (e.g. MarR). For example, antisense nucleic acids which bind to *marA* and prevent transcription or translation thereof would function as inhibitors of *marA* and agents which bind *marA*. Agents which bind to a protein expression product of a gene locus include antibodies. Inhibitors of the foregoing gene loci and expression products also include molecules which bind to the gene loci and expression products as described above. Other classes of agents and inhibitors of these types will be known to those of skill in the art.

Classes of inhibitors of efflux pumps useful in the methods and compositions of the invention have been described previously in PCT published patent application W096/33285 (including L-phenylalanyl-L-arginyl-β-naphthylamide). Methods for testing compounds for efflux pump inhibition are also described therein. Other useful inhibitors include ethanol (concentrations of about 4%), methanol, hexane and minocycline. Still other inhibitors include antisense nucleic acids and ribozymes directed against the gene(s) encoding the efflux pump. For example, antisense nucleic acids which bind to *acrAB* genes and prevent transcription or translation thereof would function as inhibitors of *acrAB*. Antibodies which bind efflux pumps or proteins which, regulate the expression of efflux pumps are another class of inhibitors. Still other inhibitors include genes which repress expression of the efflux pumps or regulatory loci (such as *marR)* which regulate expression of efflux pumps. Increasing the amount of such genes or the expression products thereof reduces the expression of efflux pumps in bacteria.

As mentioned above, the invention embraces antisense nucleic acids, including oligonucleotides, that selectively bind to a nucleic acid molecule encoding an efflux pump (e.g. *acrA)* or a molecule which regulates expression of an efflux pump (e.g. *marA)*. As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an RNA transcript

of that gene and. thereby, inhibits the transcription of that gene and/or the translation of that RNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected. including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions. i e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions. Based upon the nucleic acid sequence of a gene of interest. one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition. such antisense oligonucleotides should comprise at least 10 and. more preferably. at least 15 consecutive bases which are complementary to the target. although in certain cases modified oligonucleotides as short as 7 bases in length have been used successfully as antisense oligonucleotides (Wagner et al.. *Nature Biotechnol.* 14:840-844. 1996). Most preferably. the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of the gene or RNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation. transcription initiation or promoter sites. In addition. 3'-untranslated regions may be targeted. In addition. the antisense is targeted. preferably. to sites in which RNA secondary structure is not expected and at which proteins are not expected to bind.

In one set of embodiments. the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides. ribonucleotides. or any combination thereof. That is. the 5' end of one native nucleotide and the 3' end of another native nucleotide may be covalently linked. as in natural systems. via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by standard methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments. however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is. the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (l) at least two of its nucleotides are covalently linked via a

synthetic internucleoside linkage (i.e.. a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates. alkylphosphonates. phosphorodithioates. phosphate esters, alkylphosphonothioates. phosphoramidates. carbamates. carbonates. phosphate triesters, acetamidates. carboxymcthyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example. modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition. modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention. thus. contemplates preparations containing modified antisense molecules that are complementary to and hybridizable with. under physiological conditions. nucleic acids encoding *mar/sox/rob* or efflux pump polypeptides. together with one or more carriers.

As described above. thc invention further embraces the use of antibodies or fragments of antibodies having the ability to selectively bind to efflux pumps. as well as polypeptides which regulate the expression of efflux pumps. Antibodies include polyclonal and monoclonal antibodies. prepared according to conventional methodology.

Significantly. as is well-known in the art, only a small portion of an antibody molecule. the paratope. is involved in the binding of the antibody to its epitope (see. in general. Clark. W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons. Inc.. New York: Roitt. I. (1991) Essential Immunologv. 7th Ed., Blackwell Scientific Publications. Oxford). The pFc' and Fc regions. for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved. or which has been produced without the pFc' region. designated an $F(ab')_2$ fragment. retains both of the antigen binding sites of an intact antibody. Similarly. an antibody from which the Fc region has been enzymatically cleaved. or which has been produced without the Fc region. designated an Fab fragment. retains one of the antigen binding sites of an intact antibody molecule. Proceeding further. Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitopebinding ability in isolation. Any of the foregoing antigen

fragments are useful in the methods and compositions of the invention. The present invention also includes so-called single chain antibodies and intracellular antibodies.

Examples

Example 1:

Pine oil is a disinfectant used in products designed for household use. The possibility that such products might select for antibiotic resistance was investigated. In initial studies of a formulation whose active ingredient was pine oil, mutant colonies of *Escherichia coli* sometimes grew within the zone of inhibition surrounding a 6 mm absorbent paper disc impregnated with the product and placed upon a bacterial lawn. These and other pine oil-resistant mutants were tested for resistance to multiple antibiotics, and the genetic basis for the observed phenotype was examined.

Selection of mutants resistant to Pine-Sol/pine oil.

Mutants resistant to Pine-Sol/pine oil were obtained from stationary phase LB broth cultures of *E. coli* strain "WEC" (wild type strain 15-5068 from Carolina Biological Supply Co., Burlington, NC) and AG100 (George and Levy, *J. Bacteriol.* 155:531-540, 1983), at 30°C on nutrient agar (NP3.5GP) or LB agar with 2-3 days' incubation in a variety of ways: using the 6mm disc method: plating cells on plates or gradient plates (Curiale and Levy, *J. Bacteriol.* 151:209-215, 1982), containing PINE-SOL™ (product of Clorox Co., Oakland, CA; contains pine oil [the active ingredient], isopropanol and surfactants) or pine oil itself (obtained from the White Cap Co., Lester, PA.) All mutants were single step isolates and occurred at a frequency of about 10^{-8}.

Resistance to antibiotics of mutants selected on Pine-Sol or pine oil.

Antibiotic susceptibility was measured at 30°C using antibiotic susceptibility discs (Carolina Biological), gradient plates with the drug in the top agar (Curiale and Levy, 1982), or agar dilution plates (concentration steps of 1.5 fold; inocula of 10^5 cells/ 5 ,µl spot). Mutant NP3.5GP, selected on Pine-Sol from strain WEC, was more resistant than the parent strain to tetracycline, ampicillin, and chloramphenicol (Table 1A). Nalidixic acid was included in further tests of 11 independent mutants from strain AG100. While there was a variability of resistance phenotypes, all Pine-Sol/pine oil-selected mutants were also multidrug resistant. For genetic studies we chose three mutants of AG100 which were resistant to all four antibiotics (Table IB): AP1 and AP5 (selected on pine oil), and APS3 (selected on Pine-Sol).

Table 1. Susceptibility of Pine-Sol/pine oil and Mar mutants to Pine-Sol and to antibiotics[2]

	Strain	Characteristics	Susceptibility				
A.	WEC derivative		By discs: diameter of clearing (mm)				
			Ap	Cm	Tc		
	WEC	wild type	22	27	21		
	NP3.5GP	mutant of WEC selected on Pine-Sol gradient (0 - 1.5%)	12	11	14		
B.	AG100		By gradient plates (MIC)[b] (% by volume)	(µg/ml)			
			PS	Ap	Cm	Nal	Tc
	AG100	wild type	0.9	<1.2	2.6	1.7	1.8
	API	mutant of AG100 selected by pine oil on disc	>3.6	3.0	7.8	9.7	2.4
	AP5	mutant of AG100 selected as for API	>2.9	7.2	21	7.5	4.5
	APS3	mutant of AG100 selected on Pine-Sol gradient (0 - 1.5%)	1.8	7.7	>35	8.6	5.3
	AG102 [I][d]	Mar mutant of AG100, selected on Tc (2 steps)	>4.1	8.5	>35	14.0	>12.8

HHI80[2][d]	deletion of 39 kb including *mar* locus; has *zdd*-230::Tn*9* (Cm^R): in host strain MM294	0.3	<0.6	ND[c]	<1.8	<0.6
HHI88[2[d]]	HHI80 containing pHHM183 (*mar+*)	0.9	<1.0	ND[c]	3.7	1.2
HH191 [2][d]	HHI80 containing pHHM191 (*marR2*)	2.3	5.4	ND[c]	9.1	8.2
HH193 [2][d]	HHI80 containing pHHM193 (*marR5*) [3][d]	3.2	5.9	ND[c]	10.9	>11.4

a Abbreviations: PS (Pine-Sol), Ap (ampicillin), Cm (chloramphenicol), Nal (nalidixic acid), Tc (tetracycline).

b Gradient plate values were the averages of two to four experiments, except in the case of chloramphenicol, which involved a single determination.

c Host strain is Cm^R due to Tn*9*, so values were not determined (ND).

d References: 1) George et al., 1983; 2) Cohen et al., 1993; 3) Seoane and Levy, 1995.

Role of *mar*, *soxRS*, and *robA* loci.

Mutations in the repressor gene *marR* or in its operator *marO* in the *E. coli marRAB* operon (Ariza et al., *J. Bacteriol.* 176:143-148, 1994; Maneewannakul and Levy, *Antimicrob. Agents Chemother.* 40:1695-1698, 1996; Seoane and Levy, *J. Bacteriol.* 177:3414-3419, 1995) lead to enhanced *marA* expression and multiple antibiotic resistance. In host strain HH180, deleted of the entire *mar* region, plasmids pHHM188, pHHMl91, and pHHM193 each contain a cloned 9 kb fragment including the entire *mar* locus. In pHHM188 the *mar* locus was wild type, while in the latter two plasmids, *marR* was mutant, resulting in a Mar phenotype (Cohen et al., *J. Bacteriol.* 175:1484-1492, 1993). These Mar mutants, as well as AG102 (bearing a chromosomal *marR* mutation (Cohen et al., 1993), were resistant to Pine-Sol (Table 1 B) and to 100% pine oil (assayed by discs on MacConkey agar, data not shown) when compared to their respective wild type strains. The *marCORAB* locus was deleted in the Pine-Sol/pine oil mutants and in AG102 by P1 transduction (Provence and Curtiss, p.317-347. *In*

Gerhardt et al.. eds.. Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington. D. C., 1994) using AG 100/Kan (Maneewannakul and Levy. 1996) as the donor strain and selecting on kanamycin. The deletion caused a 60-70% reduction in the resistance of mutants to Pine-Sol (Table 2), down to approximately a wild type level. The same was true for mutant NP3.5GP (data not shown).

Table 2. Effect of inactivation of *mar. sox. rob. or acr* locus upon susceptibility to Pine-Sol

Strain	Relative MIC for Pine-Sol[a]			
	mar[b]	*sox*[b]	*rob*[b]	*acr*[b]
AG100	1	0.9	0.8	<0.06
AP1	0.5	<0.6	0.5	<0.02
AP5	0.4	0.9	0.8	<0.02
APS3	0.4	1	1	<0.04
AG102	0.4	1	1	<0.03

[a] Relative MIC is the MIC of the inactivated strain divided by the MIC of the strain before inactivation. Values in bold face indicate notable increases in susceptibility. Values obtained from both gradient plate and agar dilution experiments were averaged.

[b] Inactivated locus

Northern blot analysis for expression of *marA* mRNA in the absence and presence of the inducer salicylate (Cohen et al.., *J. Bacteriol.* 175:7856-7862. 1993) was performed. Strains were grown at 30°C in the absence (-) or presence (+) of 5 mM sodium salicylate and lysed in 3.4% SDS. 50 mM Tris-HCl. 50 mM NaEDTA. pH 8. RNA was prepared using a CsCl method as described (Ausubel et al.., eds. *Current Protocols in Molecular Biology.* John Wiley & Sons. 1996), except that no guanidine was used and an acid phenol/chloroform step was added prior to the alcohol precipitation. After gel electrophoresis on 1.5% agarose and blotting onto a Nytran Plus membrane (Schleicher and Schuell. Keene. NH), the blot was incubated with a ^{32}P[α-dCTP]-labeled 387 bp PCR *marA* probe. The hybridization signal was visualized by a PhosphorImager and processed by a linear contrast setting using ImageQuant software (both from Molecular Dynamics. Sunnyvale. CA). AP1, AP5. NP3.5GP are Pine-Sol/pine oil mutants. AG100 is wild type. AG102 is a Mar mutant. The arrow indicates the *marRAB* transcript. A white asterisk marks an artifactual spot covering both AG102 lanes. The northern blot revealed that. like Mar mutant AG102. mutants AP5 and NP3.5GP showed an overexpression of *marA* that was

enhanced by salicylatc (Fig. 1). Overexpression was also seen in mutant APS3 (data not shown). The wild type AG100 and the pine oil mutant AP1 showed no detectable signal (Fig. 1). We concluded that AP5, NP3.5GP, and APS3, but not AP1, were probably Mar mutants.

Overexpression of *soxS* and *robA*, two other regulatory genes with homology to *marA*, can also lead to multiple antibiotic resistance (Ariza et al., 1994; Ariza et al., *J. Bacteriol.* 177:1655-1661, 1995; Greenberg et al., *J. Bacteriol.* 173:4433-4439, 1991; Martin et al., *J. Bacteriol.* 178:2216-2223, 1996; and Nakajima et al., *Appl. Environ. Microbiol.* 61:2302-2307, 1995). We inactivated the *soxRS* and *robA* loci in the Pine-Sol/pine oil and Mar mutants via P1 transduction, using as donor strains DJ901, bearing a deletion in *soxRS* very closely linked to zjc2204::Tn*10*Km (Ariza et al., 1995; Greenberg et al., *Proc. Natl. Acad. Sci. USA* 87:6181-6185, 1990), and RA4468, which has a Kan insertion in *robA* (Ariza et al., 1995). The inactivations of *sox* or *rob* caused decreased resistance to Pine-Sol only in the mutant AP1 (Table 2). However, mutant API did not overexpress *soxRS* (data not shown: the probe was a 432 bp *Eco*RI-*Hind*III fragment from pSXS (Amabile-Cuevas and Demple, *Nucleic Acids Res.* 19:4479-4484, 1991), and the constitutive overexpressing strain JTG1078 (Greenberg et al., 1991) was a positive control).

Role of thc a*crAB* locus.

We deleted the *acrAB* locus in wild type and mutant strains using P1 transduction, using strain JZM120 (bearing a Kan cassette replacing most of *acrA* and half of *acrB* (Ma et al., 1995; Okusu et al., *J. Bacteriol.* 178:306-308, 1996)) as the donor. Deletion of the *acrAB* locus produced a dramatic increase in the susceptibility to Pine-Sol in all strains (Table 2). Since AP1 was affected by inactivation at *mar*, *sox*, *rob* and *acr*, yet did not overexpress *sox* or *mar*, this mutant may have a mutation in *rob*; alternately it may have a mutation in *acrR* (leading to overexpression of *acrAB* [Okusu et al., 1996]) that requires that the wild type *mar*, *sox* and *rob* loci be intact for the full resistance phenotype.

Pine-Sol/pine oil and Mar mutants showed no resistance to household disinfectants containing hydrogen peroxide, hypochlorite, alkyl dimethyl benzyl ammonium chloride (a quaternary amine), or chloroxylenol (a phenol) as their active ingredients (data not shown). However, deletion of *acrAB* (but not of *mar)* caused more than a ten fold increase in the susceptibility of strains to the products containing the quaternary amine or chloroxylenol (data not shown), suggesting that AcrAB was also involved in effluxing those two disinfectants.

EXAMPLE 2

Materials and Methods:

Bacterial strains and plasmids. The *E. coli* strains, plasmids. and their relevant properties are listed in Table 3. Unless otherwise noted, bacteria were grown and maintained at 30°C in LB broth or LB agar plates with or without the appropriate antibiotics for selection. *E coli* strains AG100-A. AG 100-B, and AG102-A. were kindly provided by H. Nikaido (Okusu et al., *J. Bacteriol.* 178:306-308, 1996).

Table 3. Bacterial strains and plasmids

E. coli Strains	Description	Source
AG100	Wild type *E. coli K-12.*	George and Levy. 1983
AG100-A	AG100: *ΔacrAB*	Okusu et al., 1996
AG100-B	AG100; *acrR* mutant	Okusu et al., 1996
AG102	*mar*R1 mutant of AG100 selected on tetracycline.	Cohen et al., 1993 George and Levy. 1983
AG102-A	AG102: Δ*acrAB*	Okusu et al., 1996
MCH164	AG100 with 39kb deletion from 33.6-34.3 min including the *mar* locus: *zdd*-230:Tn9 from which Tn*9* was spontaneously lost.	McMurry et al., 1994
AG100K	Derivative of AG100 in which a kanamycin resistance cassette has replaced most of the *mar* locus.	Maneewannakul and Levy, 1996

GC4468	Wild type *E. coli* K-12.	Greenberg et al., 1990; Touati. *Infect. Immun.* 58:1124-1128. 1983.
DJ901	GC4468 from which *soxRS901* has been deleted. *(soxRSΔ901)*::Tn*10*Km^r	Greenberg et al., 1990
RA4468	GC4468 in which a kanamycin resistance cassette was inserted into the *rob* locus. *RobA*:: *Kan*	Ariza et al., 1995
JHC1096	GC4468 in which the *mar* locus deletion (as in MCH164) has been introduced. Tn9Km^r	Ariza et al.. 1994; Greenberg et al.. 1990
JHC1098	GC4468 in which *mar* and *soxRS* deletions of DJ901 and JHC1096 have been introduced Tn*10*Km^r	Ariza et a.. 1995; Greenberg et al.. 1990
JHC1069	GC4468 bearing *cfxB1*: MarR mutant	Ariza et a.. 1995; Greenberg et al.. 1990
JTG1078	GC4468:*soxR105*. Tn*10*Km^r	Greenberg et al.. 1991
Plasmids	**Description**	**Source**
pMAK705	Temperature sensitive. low copy-number cloning vector; Cml^r.	Hamilton et al., *J. Bacteriol.* 173: 2888-2894, 1989

pMAK-TU1	pMAK705 with a 1 kb chromosomal insertion containing *marO* and *marC*. [nt 569-1577 of the *mar* locus (9)].	Goldman et al., 1996
pMAK-TU2	pMAK705 with 1.28 kb chromosomal insertion [nt 1311-2592 of *mar* locus (9)] containing *marO* and *marRAB* [derived from pHHM 193 (9)] which contains a *marR5* mutation and constitutively expressing the *marRAB* operon..	Goldman et al., 1996
pMAK-TUl&TU2	pMAK705 with 2.4 kb chromosomal insertion [nt 163-2592 of the *mar* locus (8)] containing *marC* and *marRAB* [derived from pHHM193(9)] which contains a *marR5* mutation and constitutively expresses the *marRAB* operon.	Goldman et al., 1996
pSE380	*trc* promoter expression vector; high copy number. IPTG inducible; Amp^r	Amabile-Cuevas and Demple
pSRob	926-bp *SalI-SacI* fragment containing the entire *rob* gene from pBt35-13 inserted into pSE380.	Ariza et al., 1995

pSXS	423-bp *EcoRI-HindIII* PCR fragment containing the entire *soxS* gene inserted into pSE380.	Amabile-Cuevas and Demple 1991.
pSMarAB	699-bp *EcoR1-PST1* PCR fragment [(ntl 893-2592 of *mar* locus (9)] containing *marAB* inserted into pSE380	Herein

Chemicals. Organic solvents were obtained from Aldrich Chemical Co (Milwaukee, WI). Antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO). Isopropyl-β-D-thiogalactoside (IPTG) was obtained from Alexis Corporation (San Diego, CA).

DNA manipulations. Plasmid DNA was prepared using the Promega Wizard™ Prep Kit (Madison, WI). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) and used under conditions suggested by the supplier. PCR amplification was carried out using the Perkin Elmer Cetus DNA thermal cycler 480. *Taq* polymerase and reagents were provided by Perkin Elmer Cetus and used as directed. Primers were created which flanked the coding sequence and allowed amplification of *marAB* (1893-2592 bp of the published sequence, Cohen et al., *J: Bacteriol.* 175: 1484-1492, 1993). This PCR amplicon was approximately 699 bp in size. Restriction endonuclease sites for *EcoR1* and *PST1* were incorporated into the ends of the PCR primers to ensure that insertion of fragments was in the correct orientation when cloned into pSE380, a high copy number expression vector (Invitrogen, Carlsbad,CA). The resulting plasmid construct was called pSMarAB.

DNA fragments from low melting point agarose gels, as well as PCR products, were purified using the Qiagen gel extraction kit (Qiagen Inc., Chatsworth, CA). DNA transformation was performed using the $CaCl_2$ procedure as previously described (Cohen et al., 1993) and pSE380 clones were selected using LB agar plates containing ampicillin (100 μg/ml), IPTG (0.1 mM), and X-gal (40 μg/ml).

Organic solvent tolerance assays. Organic solvent tolerance of bacterial strains grown to late logarithmic phase was measured in cultures diluted to a concentration of approximately 10^7 cells/ml. A 5 μl aliquot of the bacterial suspension was plated on LB agar and allowed to dry. An organic solvent was overlaid to a depth of 2-3 mm. The plate was sealed and incubated overnight at 30°C (Aono et al., *Biosci. Biotech. Biochem.* 59:213-218, 1995). Cyclohexane, *n*-hexane or *n*-pentane were used as

organic solvents. IPTG was added to the agar plates at a concentration of 0.5 mM when induction of plasmid genes was required. For efficiency of plating experiments (EOP), logarithmic phase cultures were diluted to OD_{530} 0.2 and 100 µl aliquots of cells from serial dilutions were spread onto LB agar plates. As mentioned above, organic solvent was then overlaid to a depth of 2-3 mm with the plate sealed and incubated overnight at 30°C. Platings were done in duplicate and colonies were counted. Growth was recorded as confluent (++), visible (<100 colonies; +), or no growth (-).

RESULTS:

The organic solvent tolerance of the *E. coli* K-12 strain AG100 was compared to an isogenic strain that constitutively expressed the *mar* operon due to a mutation in *marR*
(AG102) (Cohen et al., 1993). AG100 grew in the presence of *n*-hexane only; AG102 grew in the presence of *n*-hexane, cyclohexane (Table 4), and *n*-pentane (data not shown). Thus, constitutive expression of the *mar* locus changed the index solvent from *n*-hexane (log *P*ow 3.9) to *n*-pentane (log *P*ow 3.3)

Table 4. Organic solvent tolerance of wild-type and *mar* strains bearing *mar*, *soxS*, or *robA* plasmids.

Strain	Plasmid[b]	Growth in presence of organic solvent[a]	
		n-hexane (3.9)[c]	cyclohexane (3.4)
AG102 *(marR* mutation)		++	++
AG100 (wild-type)		++	-
AG100	pMAK-TU1	++	-
AG100	pMAK-TU2	++	+
AG100	pMAK-TU1 & TU2		
AG100	pSMarAB	++	+
AG100	pSXS	++	++
AG100	pSRob	++	++
AG100K *(marCORAB::kan)*		+	-
AG100K	pMAK-TU1	+	-
AG100K	pMAK-TU2	++	+

AG100K	pMAK-TU1 & pMAK-TU2	++	++
AG100K	pSMarAB	++	+
AG100K	pSXS	++	++
AG100K	pSRob	++	++
MCH164 *(Δmar)*		+	-
MCH164	pMAK-TU1	+	-
MCH164	pMAK-TU2	++	-
MCH164	pMAK-TU1 & pMAK-TU2	++	++
MCH164	pSMarAB	++	-
MCH164	pSXS	++	-
MCH164	pSRob	++	-
AG100-B *(acrR* mutant)		++	-
AG100-A *(ΔacrAB)*		-	-
AG102-A *(marRI. ΔacrAB)*		-	-
AG102-A	pSMarAB	-	-
AG102-A	pSXS	-	-
AG102-A	pSRob	-	-

[a](++) signifies confluent growth: (+) visible growth(< 100 colonies); (-) signifies no growth. [b]IPTG was added to plates at a concentration of 0.5mM when induction of plasmid genes was required (pSE380 derivatives).

[c]Values in parentheses are log *P*ow.

In the wild type *E. coli* AG100 background, overexpression of *marA* (on plasmid pSMarAB or pMAK-TU2) or *soxS* (on pSXS) or *robA* (on pSRob) resulted in cyclohexane tolerance (Table 4). *marC* by itself (pMAK-TU1) had no effect on cyclohexane tolerance, however, introduction of *marCORAB* on the low copy plasmid pMAK705 (pMAK-TUl&TU2) resulted in cyclohexane tolerance (Table 4).

When the *mar* locus was inactivated by replacement with a kanamycin resistance cassette (AG100K) (Maneewannakul and Levy, 1996), the strain became hypersusceptible to *n*-hexane as compared to the wild type strain (Table 4). MCH164 [a derivative of AG100 from which 39 kb of chromosomal DNA including the entire *mar* locus had been deleted (Goldman et al.. *Antimicrob. Agents Chemother.* 40:1266-1269, 1996; McMurry et al..*Antimicrob. Agents Chemother.* 38:542-546, 1994)] was. as

expected. also hypersusceptible to organic solvents (Table 4). Expression *in trans* of *marA*, *soxS*, or *robA* in AG100K, restored *n*-hexane tolerance, and increased cyclohexane tolerance in the cell (Table 4). Expression in *trans* in AG100K of *marA*, specified from plasmid pMAK-TUl&TU2 restored *n*-hexane tolerance and produced higher cyclohexane tolerance (Table 4). The better effect by the latter plasmid, over those containing only *marA*, could be linked to greater production of MarA protein from this plasmid [as visualized by antibody to MarA]. While introduction of either *marA*, *soxS*, *or robA* restored *n*-hexane tolerance in MCH164, only pMAK-TU1&TU2 produced cyclohexane tolerance in this larger deletion mutant (Table 4).

Since *acrAB* deletion dramatically decreased multiple drug resistance in Mar mutants (Okusu et al., 1996), we examined its possible role in organic solvent tolerance. Overexpression of *acrAB*, because of a mutation in *acrR* in AG100-B, enabled the strain to grow in the presence of cyclohexane (Table 4). Deletion of *acrAB* from wild-type AG100 (AG100-A) resulted in *n*-hexane sensitivity (Table 4). Deletion of *acrAB* from the Mar mutant AG102 (AG102-A) resulted in both *n*-hexane and cyclohexane sensitivity. Expression of *marA*, *soxS*, or *robA* in AG102-A failed to restore organic solvent tolerance, further demonstrating the critical role of *acrAB* (Table 4).

We next investigated a series of isogenic strains in which *sox*, *mar*, and *robA* were either overexpressed, deleted, or inactivated (Table 5). *E coli* strains overexpressing MarA (JHC1069; *cfxBl*/MarR mutation) or SoxS (JTG1078; *soxR*105 mutation) grew in the presence of both *n*-hexane and cyclohexane, whereas the wild-type GC4468 only grew in the presence of *n*-hexane (Table 5). Much like the situation in AG100, introduction of either pSMarAB, pMAK-TU2, pMAK-TUl&TU2, pSXS, or pSRob into GC4468 produced cyclohexane tolerance. Inactivation of *robA* by insertion of a kanamycin cassette (RA4468) caused *n*-hexane susceptibility (Table 5). Introduction of either *marA* (on pMAK-TU1 &TU2, pMAK-TU2, or pSMarAB), SoxS (on pSXS), or RobA (on pSRob), into the *robA* inactivated strain, increased both *n*-hexane and cyclohexane tolerance (Table 5). Deletion of *soxRS* (DJ901) had little effect on *n*-hexane tolerance (Table 5). Introduction of *marA*, *soxS*, or *robA* into the Δ*soxRS* strain produced cyclohexane tolerance (Table 5). In all these complementations, the effect of *marA* was best noted from plasmid pMAK-TU1 &TU2.

Table 5. Organic solvent tolerance of wild-type, Δ*soxRS*, or *robA::Kan*.strains bearing *mar*, *soxS*, or *robA* plasmids.

Strain (3.4)	Plasmid[b]	Growth in presence of organic solvent[a] *n*-hexane (3.9)[c]	cyclohexane
GC4468 (wild-type)		++	-
JHC1069 *(cfxB1)*		++	++
JTG1078 *(soxR105)*		++	++
GC4468	pMAK-TU1	++	-
GC4468	pMAK-TU2	++	+
GC4468	pMAK-TU1 & TU2	++	++
GC4468	pSMarAB	++	+
GC4468	pSXS	++	++
GC4468	pSRob	++	++
RA4468 *(robA::kan)*		+	-
RA4468	pMAK-TU1	+	-
RA4468	pMAK-TU2	++	+
RA4468	pMAK-TU1 & TU2	++	++
RA4468	pSMarAB	++	+
RA4468	pSXS	++	++
RA4468	pSRob	++	++
DJ901 *(ΔsoxRS)*		++	-
DJ901	pMAK-TU1	++	-
DJ901	pMAK-TU2	++	+
DJ901	pMAK-TU1 & TU2	++	++
DJ901	pSMarAB	++	+
DJ901	pSXS	++	++
DJ901	pSRob	++	++

[a](++) signifies confluent growth; (+)visible growth(<100 colonies); (-) signifies no growth. [b]IPTG was added to plates at a concentration of 0.5mM when induction of plasmid genes was required (pSE380 derivatives). [c]Values in parenthese are log *Pow*.

Since the Mar mutant strain AG102 grew confluently in the presence of cyclohexane and the AG100-*acrR* mutation strain (AG100-B) grew less well, we tried a different method, namely an efficiency of plating (EOP) to compare growth in the presence of cyclohexane (Table 6). AG102 had a greater cyclohexane EOP (73 %) compared to AG100-B (EOP of 13 %). The 6 fold difference confirmed the spot test results.

Table 6. Effect on *E. coli* cyclohexane tolerancc of a *marR* or *acrR* mutation.

Strain	Control Plate[a]	Cyclohexane Plate	EOP[b]
AG102 MarR mutant	182±5	133±7	0.73
AG100-B AcrR mutant	196±10	25±4	0.13

[a]Control LB and cyclohexane layered LB agar plates are averages of two plates.

[b]Efficiency of plating experiment (EOP) comparing growth on cyclohexane overlaid versus control plates; 10^{-6} dilution plated out and colonies counted.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference.

We claim:

Claims

1. A method for inhibiting the selection or propagation of a bacterial mutant that overexpresses an efflux pump comprising:

contacting bacteria with an agent that binds to a gene locus or an expression product thereof. wherein the expression of the gene locus enhances expression of the efflux pump, in an amount effective to inhibit the gene locus-enhanced expression of the efflux pump.

2. The method of claim 1, wherein the gene locus is selected from the group consisting of a *mar* locus. a *sox* locus and a *rob* locus.

3. The method of claim 2, wherein the gene locus is *marA*.

4. The method of claim 2, wherein the gene locus is *soxS*.

5. The method of claim 2, wherein the gene locus is *robA*.

6. The method of claim 1, wherein the efflux pump is *acr*-like.

7. The method of claim 6, wherein the efflux pump is *acrAB*.

8. The method of claim 1, wherein the agent is selected from the group consisting of antisense nucleic acids. antibodies. ribozymes. chemicals and proteins which repress expression of the gene locus.

9. The method of any of claims 1-8, wherein the agent is an antisense nucleic acids.

10. A method for rendering bacterial cells more susceptible to a non-antibiotic bactericidal or bacteriostatic agent that is a substrate of an efflux pump comprising:

administering to the bacterial cell an inhibitor of a gene locus or an expression product thereof, wherein the expression of the gene locus enhances expression of an efflux pump.

11. The method of claim 10. wherein the gene locus is selected from the group consisting of a *mar* locus, a *sox* locus and a *rob* locus.

12. The method of claim 11, wherein the gene locus is *marA*.

13. The method of claim 11. wherein the gene locus is *soxS*.

14. The method of claim 11. wherein the gene locus is *robA*.

15. The method of claim 10. wherein the efflux pump is *acr*-like.

16. The method of claim 15. wherein the efflux pump is *acrAB*.

17. The method of claim 10. wherein the inhibitor is selected from the group consisting of antisense nucleic acids. antibodies. ribozymes. chemicals and proteins which repress expression of the gene locus.

18. The method of any of claims 10-17. wherein the inhibitor is an antisense nucleic acid.

19. The method of any of claims 10-17. wherein the inhibitor is L-phenylalanyl-L-arginyl-β-naphthylamide.

20. A method for rendering bacterial cells more susceptible to a non-antibiotic bactericidal or bacteriostatic agent that is a substrate of an efflux pump comprising:

administering to the bacterial cell an inhibitor of the efflux pump.

21. The method of claim 20. wherein the efflux pump is *acr*-like.

22. The method of claim 21. wherein the efflux pump is *acrAB*.

23. The method of claim 20. wherein the inhibitor is selected from the group consisting of L-phenylalanyl-L-arginyl-β-naphthylamide, 4% ethanol, X% methanol, hexane, minocycline.

24. The method of any of claims 20-23, wherein the inhibitor is L-phenylalanyl-L-arginyl-β-naphthylamide.

25. A method for increasing the ability of bacterial cells to survive in an organic solvent comprising:

enhancing expression in the bacterial cells of an organic solvent bacterial efflux pump by growing the bacterial cells in the presence of a non-*mar/sox/rob* agent that induces the overexpression of the organic solvent bacterial efflux pump.

26. The method of claim 25, wherein the agent is a gene encoding an *acr*-like pump or an expression product thereof.

27. The method of claim 26, wherein the *acr*-like pump is *acrAB*.

28. The method of claim 25, wherein the agent is selected from the group consisting of an antibiotic, and a non-antibiotic antibacterial compound.

29. A method for decreasing the ability of bacterial cells to survive in an organic solvent comprising:

reducing expression in the bacterial cells of an organic solvent bacterial efflux pump by growing the bacterial cells in the presence of an agent that reduces the expression of the organic solvent bacterial efflux pump.

30. The method of claim 29, wherein the agent is an inhibitor of a gene locus or an expression product thereof, wherein the expression of the gene locus enhances expression of an efflux pump.

31. The method of claim 30 wherein the gene locus is selected from the group consisting of a *mar* locus, a *sox* locus and a *rob* locus.

32. The method of claim 31 wherein the gene locus is *marA*.

33. The method of claim 31 wherein the gene locus is *soxS*.

34. The method of claim 31 wherein the gene locus is *robA*.

35. The method of claim 30 wherein the efflux pump is *acr*-like.

36. The method of claim 35 wherein the efflux pump is *acrAB*.

37. The method of claim 30 wherein the inhibitor is selected from the group consisting of antisense nucleic acids, antibodies, ribozymes, chemicals and proteins which repress expression of the gene locus.

38. The method of any of claims 29-37 wherein the inhibitor is an antisense nucleic acid.

39. The method of any of claims 29-37, wherein the inhibitor is L-phenylalanyl-L-arginyl-β-naphthylamide.

40. A method for testing the ability of a non-antibiotic composition to induce a multiple antibiotic resistance phenotype in a bacterium comprising

(a) contacting the bacterium with the non-antibiotic composition,

(b) determining the expression of a bacterial gene locus, the altered expression of which is indicative of (correlated with) induction of the multiple antibiotic resistance phenotype in the bacterium, and

(c) comparing the result of (b) with a control, wherein altered expression of the bacterial gene locus indicates that the non-antibiotic composition induces the multiple antibiotic resistance phenotype in the bacterium

41. The method of claim 40, wherein the gene locus is selected from the group consisting of a *mar* locus, a *sox* locus, a *rob* locus and an *acr*-like efflux pump locus.

42. The method of claim 41, wherein the gene locus is *mar*A.

43. The method of claim 41, wherein the gene locus is *soxS*.

44. The method of claim 41, wherein the gene locus is *robA*.

45. The method of claim 41, wherein the efflux pump is *acr*-like.

46. The method of claim 45, wherein the efflux pump is *acrAB*.

47. The method of 40, wherein the composition is an inactive ingredient.

48. The method of claim 47, wherein the inactive ingredient is a non-bactericidal ingredient.

49. The method of claim 47, wherein the inactive ingredient is a non-bacteriostatic ingredient.

50. The method of claim 40, wherein step (b) is performed by determining the enzymatic activity of an expression product of a marker gene fused to the bacterial gene locus.

51. The method of claim 50, wherein the marker gene is *lacZ*.

52. A composition comprising: a non-antibiotic bactericidal or bacteriostatic first agent and a second agent that inhibits the expression or activity of an efflux pump.

53. The composition of claim 52, wherein the second agent inhibits the expression of a gene locus or an expression product thereof, wherein the expression of the gene locus enhances expression of the efflux pump.

54. The composition of claim 53, wherein the second agent is selected from the group consisting of antisense nucleic acids, antibodies, ribozymes, chemicals and proteins which repress expression of the gene locus.

55. The composition of claim 54, wherein the second agent is an antisense nucleic acid.

56. The composition of claim 52, wherein the second agent inhibits an *acr*-like efflux pump.

57. The composition of claim 56, wherein the second agent is selected from the group consisting of L-phenylalanyl-L-arginyl-β-naphthylamide, 4% ethanol, X% methanol, hexane, minocycline.

58. The method of claim 57, wherein the second agent is L-phenylalanyl-L-arginyl-β-naphthylamide.

59. The composition of claim 52, wherein the first agent is selected from the group consisting of triclosan, pine oil, quaternary amine compounds including alkyl dimethyl benzyl ammonium chloride, chloroxylenol, triclocarbon, disinfectants and organic solvents.

FIG. 1