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(54) Title: PRODUCTS AND METHODS FOR IN VIVO SECRETION OF MONATIN

(57) Abstract: Products and methods for the in vivo production of monatin sweetener are provided. The products include microorganisms that are genetically modified to secrete or to improve secretion of monatin; microorganisms that are genetically modified to produce monatin; and microorganisms that are genetically modified to both secrete or improve secretion of monatin and produce monatin. The methods include producing monatin in such genetically engineered microorganisms.

PRODUCTS AND METHODS FOR *IN VIVO* SECRETION OF MONATIN

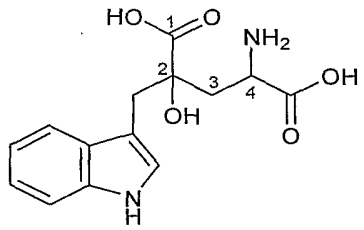
BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates generally to products and methods for the *in vivo* production of monatin sweetener. More specifically, the present invention relates to products and methods for the *in vivo* secretion of monatin sweetener.

Related Art

[0002] Monatin is a high-intensity sweetener having the chemical formula:



[0003] Monatin includes two chiral centers leading to four potential stereoisomeric configurations. The R,R configuration (the "R,R stereoisomer" or "R,R monatin"); the S,S configuration (the "S,S stereoisomer" or "S,S monatin"); the R,S configuration (the "R,S stereoisomer" or "R,S monatin"); and the S,R configuration (the "S,R stereoisomer" or "S,R monatin"). As used herein, unless stated otherwise, the term "monatin" is used to refer to compositions including all four stereoisomers of monatin, compositions including any combination of monatin stereoisomers, (e.g., a composition including only the R,R and S,S, stereoisomers of monatin), as well as a single isomeric form.

[0004] For purposes of this disclosure, the monatin carbon backbone will be numbered as illustrated above, with the carbon attached to the alcohol group being identified as the 2-position carbon and the carbon directly covalently attached to the amino group being identified as the 4-position carbon. Consequently, references herein to R,R monatin, S,S monatin, R,S monatin,

and S,R monatin mean: 2R,4R monatin, 2S,4S monatin, 2R,4S monatin, and 2S,4R monatin, respectively, unless otherwise indicated.

[0005] It should be noted that in the literature, the monatin carbon backbone has also been numbered using an alternative convention, with the carbon attached to the alcohol group being the 4-position carbon, and the carbon attached to the amino group being the 2-position carbon. Accordingly, for example, references to 2S,4R monatin in this disclosure would be the same as references to 2R,4S monatin in literature using the alternative numbering convention.

[0006] Furthermore, because of various naming conventions, monatin is known by a number of alternative chemical names, including: 2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid; 4-amino-2-hydroxy-2-(1H-indol-3-ylmethyl)-pentanedioic acid; 4-hydroxy-4-(3-indolylmethyl)glutamic acid; and, 3-(1-amino-1,3-dicarboxy-3-hydroxy-but-4-yl)indole.

[0007] At least in part because of its sweetening characteristic, it would be desirable to have an economic source of monatin. Certain isomeric forms of monatin can be found in the bark of roots of the *Schlerochiton ilicifolius* plant located in the Transvaal Region of South Africa. However, the concentration of the monatin present in the dried bark, expressed as the indole in its acid form, has been found to be about 0.007% by mass. *See* U.S. Patent No. 4,975,298. Further, the method by which monatin is produced in the plant is presently unknown. U.S. Patent Application No. 10/422,366 ("the '366 Application"), on the other hand, which is hereby incorporated by reference, discloses, *inter alia*, polypeptides, pathways, and microorganisms for *in vivo* and *in vitro* production of monatin.

BRIEF SUMMARY OF THE INVENTION

[0008] The production of monatin is thought to be an equilibrium process. In order to increase production of monatin above the equilibrium amount, and potentially make production of monatin even more economical, it would be desirable to remove products or increase the amount of substrates involved in reactions for making monatin. With respect to *in vivo* production, it could therefore be desirable to secrete monatin from the cell as a means of removing

product and pushing the equilibrium forward. For purposes of this specification, the terms "cell", "organism", and "microorganism" are used interchangeably, and include, without limitation, and unless otherwise stated, bacteria, fungi such as yeasts and molds, algae, protozoa, microbes, and viruses.

[0009] The inventors have developed a novel approach utilizing transporters—which were heretofore unknown to cooperate with monatin—for removing monatin from the cell, for example into the periplasmic space or into the surrounding medium Transporters are cell membrane proteins, which catalyze the transfer of certain solutes across one or more cell membranes. For example, bacterial species that have developed clinical resistance to antibiotics use transporters to pump the drugs or other toxic agents across the cell membrane into the medium. These efflux pumps utilize energy either from ATP hydrolysis or the proton-motive force to promote the extrusion of toxic agents. No wild-type microorganisms are presently reported to produce monatin in nature, and so similarly there were heretofore no reported transporters for secreting monatin.

[0010] According to some embodiments, a method for producing monatin (i.e. all four stereoismers of monatin or a subset thereof, including a single isomeric form) *in vivo* is provided that includes using one or more types of transporter systems to secrete monatin that is located inside a cell out into the periplasmic space or into a medium, such as a culture medium. Non-limiting examples of suitable transporter systems include the AcrAB system, the EmrAB system, and systems that include homologs of AcrAB or EmrAB. In one permutation, monatin is produced in and is at least partially secreted by a microorganism that is genetically modified to have the ability to produce monatin, and whose corresponding wild-type form includes one or more transporter systems capable of secreting monatin but itself does not produce monatin. In one permutation, monatin is produced in and is at least partially secreted by a microorganism naturally capable of producing monatin, but that is genetically modified to express or overexpress one or more transporter systems or one or more components of transporter systems capable of secreting monatin. In one permutation, monatin is produced and at least partially secreted by a microorganism that has been genetically modified to

express transporters or components of transporter systems involved with secreting monatin. In one permutation, monatin is produced and at least partially secreted by a microorganism that has been genetically modified to express components of transporters capable of secreting monatin. For example, the microorganism may be genetically modified to overexpress the channel-forming protein component of the transporter system such as AcrAB and/or EmrAB. As another example, the microorganism may be genetically modified to express components of transporters that are heterologous to the microorganism, native to the microorganism, or a combination thereof.

[0011] According to some embodiments, microorganisms are provided that have been genetically modified to express or overexpress one or more transport systems that enable directed exchange of solutes between the microorganism and its environment. In some embodiments, the microorganisms are genetically engineered to express, or overexpress, transporters that selectively transport monatin, for example over intermediates in the monatin pathway.

[0012] According to some embodiments, monatin is produced in cells which exhibit increased transporter activity as compared to an appropriate control, e.g. as described in examples herein. In one permutation, monatin is produced in a microorganism that is genetically modified to overexpress one or more types of transporter systems capable of secreting monatin. In one permutation, monatin is produced in a microorganism genetically modified to overexpress one or more components of a transporter system. For example, monatin may be produced in a microorganism that is genetically modified to overexpress the channel-forming protein component (such as AcrAB or EmrAB) of a transporter system. As another example, monatin may be produced in a microorganism that in addition, or in the alternative, overexpresses the outer membrane factor component (e.g. TolC) of a transporter system. In one permutation, monatin is produced in a microorganism exposed to an inducing compound (i.e. a compound that triggers expression of a transporter system or component of a transporter system or that stimulates secretion activity). For example, monatin may be produced in a microorganism that is provided sodium decanoate, carbonyl cyanide 3-chlorophenylhydrazone ("CCCP"), or salicylate in its growth medium. "Growth medium," "culture medium," and

"fermentation medium" are used herein interchangeably. In one permutation, the presence of inducing compounds results in monatin transport in microorganisms which do not appear to secrete monatin absent the inducing compounds. In one permutation, the presence of inducing compounds results in increased secretion of monatin by a microorganism relative to the amount secreted by an appropriate control.

[0013] According to some embodiments of the invention, methods for verifying transporter efficacy for secreting monatin are provided. In one permutation, the method includes transforming a plasmid containing the monatin operon genes into a host microorganism that has a deletion in the targeted transporter and screening for loss of monatin transport/secretion relative to wild-type controls. In one permutation, transporter genes are overexpressed by cloning them on a multi-copy plasmid, transforming a host engineered to produce monatin as described above, and screening is done for increase of monatin transport as compared to wild-type controls with no overexpression of the respective transporter genes. In one permutation, monatin secretion is evaluated by using an inducer to increase transporter activity and comparing monatin production and/or secretion to appropriate uninduced controls.

[0014] According to some embodiments, monatin is produced in a microorganism lacking one or more, transporters, for example, lacking four specific Putative Efflux Transporters identified as YhcP (AaeB), YccS, YjcQ and YhfK.

[0015] According to some embodiments, monatin is produced in a glutamate auxotroph. In one permutation, monatin is produced in a glutamate auxotroph genetically engineered to have the ability to produce monatin. In one permutation, monatin is produced in a glutamate auxotroph genetically engineered to overexpress one or more types of transporter systems capable of secreting monatin, or components of such transporter systems. In one permutation, monatin is produced in a glutamate auxotroph cultivated under fermentation conditions that increase amino acid transport.

[0016] According to some embodiments, monatin is produced in a microorganism containing a transporter system or systems capable of translocating glutamate or structurally similar molecules in exchange for

malate. In one permutation, monatin is produced by a microorganism containing a transporter system or systems capable of translocating glutamate or structurally similar molecules in exchange for malate and that has been genetically modified to have the ability to produce monatin. In one permutation, monatin is produced in a microorganism containing a transporter system or systems capable of translocating glutamate or structurally similar molecules in exchange for malate and which has been genetically engineered to be a glutamate auxotroph.

[0017] It should be apparent to one of ordinary skill in the art from reading this disclosure that specific embodiments of the present invention may encompass one, some or all of the referenced permutations and embodiments, or alternatively or in addition encompass permutations or embodiments which are not explicitly identified, but may become apparent from the disclosure herein. For example, it would be considered within the scope of the invention to induce expression of transporters using compounds that are explicitly identified in the disclosure (e.g. sodium decanoate and/or CCCP and/or salicylate) and compounds that may not be identified in this disclosure.

[0018] Similarly, embodiments in accordance with the invention may encompass combinations of permutations/embodiments not explicitly identified. For example, it would be considered within the scope of the invention to simultaneously implement multiple treatments used to individually improve monatin excretion. For example, provision of ampicillin and/or Tween 20 may be combined with provision of sodium decanoate. Example 7 provides another illustration of a suitable embodiment involving combining permutations/embodiments in accordance with the invention. In Example 7, monatin is produced in a microorganism that is both induced to express transporters by providing sodium decanoate to the growth medium and that is genetically engineered to overexpress TolC.

[0019] As should therefore be realized from the description herein the invention is capable of modifications in various aspects, all without departing from the spirit and scope of the present invention. The description, therefore, should be regarded as illustrative in nature and not restrictive and while multiple embodiments/permutations are disclosed, still other embodiments/permutations of the present invention should be apparent to

those skilled in the art from the description herein, which shows and describes illustrative embodiments/permutations of the invention. Accordingly, unless otherwise indicated, all examples are non-limiting, whether or not explicitly identified as such.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0020] **FIG. 1** is a sequence alignment showing the homology between the auxin transporter AtPGP1 and 7 different proteins, a subset of the results of a BLAST analysis of the NCBI database. These proteins are designated as Br ABB97035, St AAD10836, Sb AAR10387, Os XP483819, Os CAD59580, ZMPGP1, and AAR00316.

DETAILED DESCRIPTION OF THE INVENTION

[0021] According to some embodiments, the present disclosure provides methods and products for secreting monatin out of a microorganism, for example into the periplasmic space or into a medium, such as a culture medium. According to some embodiments, the present disclosure also provides for the development and use of transporters for secreting monatin outside a host microorganism into a desired environment, such as a medium, including a culture medium. Such transportation of monatin out of the microorganism may also increase the amount and/or rate of production of monatin relative to an appropriate control, e.g. as described in the examples herein.

[0022] As used herein, "including" means "comprising." In addition, the singular forms "a" or "an" or "the" include plural references unless the context clearly dictates otherwise. For example, reference to "comprising a protein" includes one or a plurality of such proteins, and reference to "comprising the cell" includes reference to one or more cells and equivalents thereof known to those skilled in the art, and so forth.

[0023] As used herein, the term "about" encompasses the range of experimental error that occurs in any measurement. Unless otherwise stated, all measurement numbers are presumed to have the word "about" in front of them even if the word "about" is not expressly used.

[0024] As used herein, the term "increased transporter activity" encompasses an observation that the amount and/or rate of monatin production or secretion is higher than the amount and/or rate of monatin production from the appropriate control.

[0025] As used herein, the terms "secreted" and "excreted" are used interchangeably and encompass cells that generate and separate a substance from those cells by moving that substance from within the cell into the periplasmic space or into the environment outside of the cell, such as into a surrounding medium.

[0026] As used herein, the terms "partially secreted" and "partially excreted" are used interchangeably and encompass cells that generate and separate some of a specific substance from the cell, but not all. In some aspects, the cells will maintain some of the substance within the cell.

[0027] As used herein, the terms "monatin operon gene" and "monatin operon genes" encompass gene(s) that encode one or more of the enzymes used in the synthesis of monatin.

[0028] As used herein, the terms "pump," "pumps," "pump system," "pump systems," "efflux system," and "efflux systems" are used interchangeably and encompass one or more transporters that are capable of moving a substance from the inside of a cell to a desired environment, for example, medium surrounding the cell and/or are capable of moving a substance from the area surrounding a cell to the inside of the cell.

[0029] As used herein, the term "heterologous" encompasses elements that are foreign or not native to the recited element. For example, heterologous components of a transport system encompass components that are not naturally components of that system.

[0030] As used herein, the terms "cooperate with" and "cooperates with" encompass molecule(s) that interact, covalently binds and/or regulates another molecule in a manner to achieve or enhance the functioning of that molecule. Additionally, the terms encompass molecule(s) that initiate a regulatory pathway leading to the down-stream activation or inhibition of another molecule.

[0031] As used herein, the terms "isolation," "isolating," and "isolate" encompass the process of removing a substance from its environment or host;

the process does not have to yield a substance with a certain degree of purity unless otherwise indicated.

[0032] As used herein, the term "isolated" encompasses a substance that has been removed from its environment or host; the substance does not have to be pure.

[0033] As used herein, the phrase "one or more components of a transporter system" includes the complete transport system itself.

Production of Monatin in Microorganisms

[0034] As described, *inter alia*, in WO 03/091396 A2 (*see, e.g.*, Figures 1–3 and 11-13), monatin can be produced from tryptophan through a multi-step pathway involving biological conversions (i.e. facilitating the reaction of a substrate to a product with a polypeptide). A pathway described involves biologically converting tryptophan to indole-3-pyruvate, biologically converting indole-3-pyruvate to 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid ("MP"), and biologically converting MP to monatin.

[0035] Enzymes useful for converting tryptophan to indole-3-pyruvate include members of enzyme classes ("EC") 2.6.1.27, 1.4.1.19, 1.4.99.1, 2.6.1.28, 1.4.3.2, 1.4.3.3, 2.6.1.5, 2.6.1.-, 2.6.1.1, 2.6.1.21 and 3.5.1.-. These classes include polypeptides such as: tryptophan aminotransferase, which converts L-tryptophan and α-KG (i.e., α-ketoglutarate, also called 2-oxoglutarate) to indole-3-pyruvate and L-glutamate; D-tryptophan aminotransferase, which converts D-tryptophan and a 2-oxo acid to indole-3-pyruvate and an amino acid; tryptophan dehydrogenase, which converts L-tryptophan and NAD(P) to indole-3-pyruvate and NH_3 and NAD(P)H; D-amino acid dehydrogenase, which converts D-amino acids and FAD to indole-3-pyruvate and NH_3 and $FADH_2$; tryptophan-phenylpyruvate transaminase, which converts L-tryptophan and phenylpyruvate to indole-3-pyruvate and L-phenylalanine; L-amino acid oxidase, which converts an L-amino acid and H_2O and O_2 to a 2-oxo acid and NH_3 and H_2O_2; D-amino acid oxidase, which converts a D-amino acid and H_2O and O_2 to a 2-oxo acid and NH_3 and H_2O_2; and tryptophan oxidase, which converts L-tryptophan and H_2O and O_2 to indole-3-pyruvate and NH_3 and H_2O_2. These classes also contain tyrosine (aromatic) aminotransferase, aspartate aminotransferase, D-amino acid (or D-alanine)

aminotransferase, and broad (multiple substrate) aminotransferase which have multiple aminotransferase activities, some of which can convert tryptophan and a 2-oxo acid to indole-3-pyruvate and an amino acid. In addition, these classes include phenylalanine deaminases, which can convert tryptophan to indole-3-pyruvate and ammonium in the presence of water.

[0036] Enzymes useful for converting indole-3-pyruvate to MP include members of enzyme classes 4.1.3.-, 4.1.3.16, 4.1.3.17, and 4.1.2.-. These classes include carbon-carbon synthases/lyases, such as aldolases that catalyze the condensation of two carboxylic acid substrates. Peptide class EC 4.1.3.- are synthases/lyases that form carbon-carbon bonds utilizing oxo-acid substrates (such as indole-3-pyruvate) as the electrophile, while EC 4.1.2.- are synthases/lyases that form carbon-carbon bonds utilizing aldehyde substrates (such as benzaldehyde) as the electrophile. For example, KHG aldolase (EC 4.1.3.16) and ProA aldolase (EC 4.1.3.17), are known to convert indole-3-pyruvate and pyruvate to MP. MP can also be generated using chemical reactions, such as the aldol condensations.

[0037] Enzymes useful for converting MP to monatin include members of enzyme classes: tryptophan aminotransferases (2.6.1.27), tryptophan dehydrogenases (1.4.1.19), D-amino acid dehydrogenases (1.4.99.1), glutamate dehydrogenases (1.4.1.2-4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminases (2.6.1.28), or more generally members of the aminotransferase family (2.6.1.-) such as aspartate aminotransferase (EC 2.6.1.1), tyrosine (aromatic) aminotransferase (2.6.1.5), D-tryptophan aminotransferase, or D-alanine (2.6.1.21) aminotransferase (*see* Figure 2 of WO 03/091396 A2). This reaction can also be performed using chemical reactions. Amination of the keto acid (MP) is performed by reductive amination using ammonia and sodium cyanoborohydride. Figures 11-13 of WO 03/091396 A2 show additional polypeptides that can be used to convert MP to monatin, as well as providing increased yields of monatin from indole-3-pyruvate or tryptophan.

[0038] As described herein, monatin can be produced *in vivo* using organisms genetically modified to have the ability to produce monatin, for example using the above-disclosed pathway, or by providing appropriate substrates under appropriate conditions to organisms that express the enzymes disclosed in the

above-pathway. For example, monatin can be produced using an organism whose wild-type form produces tryptophan and expresses at least one of the enzymes disclosed in the pathway elucidated in WO 03/091396 and that is genetically modified to express other enzymes not present in the wild-type form but useful in the monatin production pathway.

[0039] In accordance with some embodiments of the present invention, monatin is produced in an organism (for example, a host cell) that expresses one or more transporter systems capable of secreting monatin. According to some embodiments, monatin is produced in an organism that expresses the transporter system(s) in its wild-type form. According to some embodiments, monatin is produced in an organism that is genetically modified to express one or more transporter system(s) that are heterologous to the microorganism. According to some embodiments, monatin is produced in an organism that is genetically modified to express one or more components of the transporter systems used to transport the monatin to the external medium.

[0040] Any nucleic acid encoding a polypeptide having transporter activity can be isolated from any organism and cloned into the host organism of choice. Examples of genes encoding a polypeptide having transporter activity include, without limitation, (1) AAp and Bra genes from *Rhizobium*, (2) *Arabidopsis* AUX1 gene or PIN1 polypeptide (permease, auxin secretion), (3) MadN of *Malonomonas rubra* (an acetate efflux pump), (4) *L. lactis* organic anion transporters which are similar to mammalian multidrug resistance polypeptides and yeast Pdr12, with a specificity for anions instead of cations or hydrophobic molecules, (5) multidrug resistance polypeptide Mrp2, which can secrete non-bile organic anions from the liver, and (6) aspartate/glutamate carrier (AGC) polypeptides. The AAp and Bra genes from *Rhizobium* are general amino acid transporters primarily involved in uptake of glutamate and aspartate in bacteroids. They, however, can efflux other amino acids when heterologous amino acids are present in high levels in the media. Example 25 indicates that overexpression of the *Arabidopsis* auxin transporter gene results in increased monatin production.

[0041] Additionally, expression or overexpression of homologs to auxin transporters would be expected to also yield increased monatin production. Examples of such homologs include those containing the conserved regions of

the auxin transporters indicated in FIG. 1. For example, the homolog of the auxin transporter may comprise one or more amino acid sequences chosen from PXGKTXAXVGXSGSGKSTVVSLXERFYXPXXGXXXLDG, LXLXXLRXQIGLVXQEPXLFATXIXENXLG, and QVGERGXQLSGGQKQRIAIARAMLXXPXILLLDEATSALD, wherein X is an amino acid at the indicated position of any one of AtPGP1, BrABB97035, StAAD10836, ZmPGP1_AAR00316, SbAAR10387, OsXP_483819, OS_CAD59580, and AtPGP19 as aligned with the amino acid sequence of the homolog, as shown in FIG. 1B. Another suitable homolog of an auxin transporter may comprise one or more amino acid sequences chosen from LPXGYXTXVGERGVQLSGGQXQRIAIARA and LLDEATSALDAESEXXXQEAL, wherein X is an amino acid at the indicated position of any one of AtPGP1, BrABB97035, StAAD10836, ZmPGP1_AAR00316, SbAAR10387, OsXP_483819, OS_CAD59580, and AtPGP19 when aligned with the amino acid sequence of the homolog, as shown in FIG. 1D.

[0042] Non-limiting examples of transporters capable of secreting monatin include the AcrAB efflux system and the EmrAB efflux system. Example 1 demonstrates that the AcrAB pump is capable of secreting monatin. Example 2 demonstrates that the EmrAB pump is capable of secreting monatin. Example 3 also illustrates the capability of both the AcrAB and the EmrAB efflux systems for secreting monatin.

[0043] Based on the positive test results for AcrAB and EmrAB, it is expected that certain other transporters should be capable of secreting monatin. Generally, the AcrAB and EmrAB systems belong to a class of transporters known as multi-drug transporters. Multi-drug transporters are transporters thought to be capable of protecting cells against a wide variety of toxic molecules by active extrusion of those toxic molecules. Based on the broad overlap in the type of molecules effluxed by multi-drug transporters, and the AcrAB and the EmrAB transporter systems classification as multi-drug transporters, it is expected that other multi-drug transporters should be capable of secreting monatin. In particular, other transporters in the RND family (a subclass of the multi-drug transporters that includes AcrAB) and other transporters in the MF family (another subclass of the multi-drug transporters

but which includes EmrAB) are expected to be capable of secreting monatin. Homologs of the tested transporter are also expected to secrete monatin. A BLAST search of a microbial database, ERGO, conducted using the AcrA, AcrB, EmrA, EmrB or TolC peptides as bait, resulted in the identification of 154, 213, 231, 236 and 115 homologs, respectively.

[0044] Even more specifically, AcrEF is an efflux pump highly homologous to AcrAB, and consequently the AcrEF system are expected to be capable of secreting monatin. Example 24 provides a mutant *E. coli* strain in which the gene that encodes the AcrEF transporter system has been knocked out.

[0045] Other non-limiting examples of transporters are expected to be useful for secreting monatin because of their homology to AcrAB include:

- AcrA homologue MexA from *Pseudomonas aeruginosa*. Fernandez-Recio, J., *et al*, "A model of a transmembrane drug-efflux pump from Gram-negative bacteria," *FEBS Lett. 578*:5-9, (2004);
- Multi-drug transporter systems derived from *Neisseria gonorrhoeae*, which contains genes *mtrRCD* whose products are related to AcrRAB. Pan, W., and Spratt, B.G., "Regulation of the permeability of the gonococcal envelope by the *mtr* system," *Mol. Microbiol. 11*:769-775, (1994);
- The product of the gene *ameB,* which is homologous to members of the RND-type transporters. These include AcrB of *E.coli*, MexB, MexD and mexF of *Pseudomonas aeruginosa*, TtgB, TtgE and SrpB of *Pseudomonas putida*;
- The AcrEF efflux pump implicated in multidrug resistance with a substrate range similar to that of AcrAB (also transports novobiocin). The AcrEF system plays a significant role in indole efflux. The AcrEF efflux pump is involved in solvent resistance in *E.coli* and utilizes TolC to improve solvent resistance.
- The product of the *yegMNOB* operon which was renamed *mdtABCD*, where mdt stands for multidrug transporter. Baranova N., and Nikaido H., "The baeSR two-component regulatory system activates transcription of the *yegMNOB (mdtABCD)* transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and

deoxycholate," *J Bacteriol. 184*:4168-4176 (2002). Because AcrAB is a multidrug transporter as well and can also efflux novobiocin, the *mdtABCD* operon could also be a candidate for a monatin transporter.

- The *yhiU/V* gene products. Ma, D., *et al.*, "Genes *acrA* and *acrB* encode a stress-induced efflux of *Escherichia coli*," *Mol. Microbiol. 16*:45-55, (1995); Ma, D., *et al.*, "Efflux pumps and drug resistance in gram negative bacteria," *Trends Microbiol. 2*:489-493, (1994).
- AcrB and AcrD belong to the resistance nodulation division ("RND") superfamily and share a similar topology, which includes a pair of large periplasmic loops containing more than 300 amino acid residues each. The alterations in substrate range accompanying the exchange of loop regions is taken to imply that substrate recognition (and presumably binding) is determined largely by the two periplasmic loops. Elkins, C.A., and Nikaido, H., "Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops," *J Bacteriol. 184*:6490-6498, (2002). It is possible to make mutations in the two periplasmic loops that could have an impact on the specificity of substrates that are transported by the AcrAB transporter, to increase selectivity for monatin and decrease transport of monatin intermediates.

[0046] Non-limiting examples of transporters that also are expected to be capable of secreting monatin due to their homology with EmrAB include:

- MdeA (multidrug efflux A) is a chromosomally encoded multidrug resistance efflux protein identified in *Staphylococcus aureus*. MdeA belongs to the major facilitator superfamily and is most closely related, among known efflux proteins to EmrB from *E.coli* and LmrB from *Bacillus subtilis*. Comparison of MdeA with protein sequence databases revealed significant residue identity to hypothetical and known MDR proteins. Among the top 100 hits in a standard BLASTP search (June 2002), there were only 5 experimentally proven efflux or MDR proteins:

- LmrB of *B. subtilis* (O35018) 37% identity to MdeA (Kumano M., *et al.*, "A 32 kb nucleotide sequence from the region of the lincomycin-resistance gene (22 degrees-25 degrees) of the *Bacillus subtilis* chromosome and identification of the site of the lin-2 mutation," *Microbiology 143*:2775-2782, (1997));
- FarB of *Neisseria gonorrhoeae* (AAD54074) 24% identity to MdeA (Lee E.H., and Shafer W.M., "The farAB-encoded efflux pump mediates resistance of gonococci to long-chained antibacterial fatty acids," *Mol Microbiol. 33*:839-845 (1999));
- TcmA of *Streptomyces glaucescens* (P39886) 24% identity to MdeA (Guilfoile P.G., and Hutchinson C.R., "The *Streptomyces glaucescens* TcmR protein represses transcription of the divergently oriented *tcmR* and *tcmA* genes by binding to an intergenic operator region," *J Bacteriol. 174*:3659-3666 (1992));
- Pur8 of *Streptomyces anulatus* (P42670) 25% identity to MdeA (Tercero J.A., *et al.*, "The *pur8* gene from the pur cluster of *Streptomyces alboniger* encodes a highly hydrophobic polypeptide which confers resistance to puromycin," *Eur J Biochem. 218*:963-971 (1993)). The LmrB, TcmA, Pur8, and EmrB proteins mentioned above, confer resistance to hydrophobic compounds or antibiotics while the FarB protein confers resistance to antibacterial fatty acids;
- The QacA protein of *S. aureus*, at 23% identity to MdeA, is the closest related, functionally characterized staphylococcal efflux protein. Mitchell B.A., *et al.*, "QacA multidrug efflux pump from *Staphylococcus aureus*: comparative analysis of resistance to diamidines, biguanidines, and guanylhydrazones," *Antimicrob Agents Chemother. 42*:475-477, (1998). Multiple alignment of MdeA and these six known MDRs demonstrates conservation of many of the motifs identified in bacterial MDR proteins. Putman M., *et al.*, "Molecular properties of bacterial multidrug transporters," *Microbiol Mol Biol Rev. 64*:672-693 (2000). MdeA was predicted to have 14 TMS, and the alignment of the MdeA and QacA sequences illustrated the coincidence of the predicted and experimentally demonstrated

TMS regions, respectively. (Huang J., *et al.*, "Novel chromosomally encoded multidrug efflux transporter MdeA in *Staphylococcus aureus*," *Antimicrob Agents Chemother.* *48*:909-917 (2004).

[0047] The methods in Example 4 can be used to verify the ability of a given transporter such as those identified above for secreting monatin. Moreover, the methods of Example 4 can be used to screen for additional transporters useful for secreting monatin. A strategy for identifying transporters capable of transporting monatin, would be to apply the screening methods of Example 4 to any organism that can transport glutamate, tryptophan, indole compounds, or that has other characteristics that may be consistent with an ability to secrete monatin. Such organisms include, but are not limited to, (1) organisms having a high number of predicted secondary transporters such as *E. coli, B. subtilis, Pantoea* and *Ricksettia,* (secondary active transporters use electrochemical gradients and typically have many (>7) transmembrane regions as well as regions located in the cytoplasm and extracellular space), (2) organisms that secrete glutamate such as *Corynebacteria* and *Brevibacteria,* (3) plants and legume containing plants such as soybean, peas, peanuts, and beans, (4) *Rhizobium* species, (5) organisms that have a high resistance to acids such as lactic acid bacteria, *Acetobacter* strains, *Kluyveromyces, Saccharomyces cerevisiae,* and *Aspergillus niger,* (6) organisms that secrete indole-pyruvic acid such as S*treptomyces griseoflavus* and *P. stewartii,* (7) organisms that are GRAS (generally recognized as safe), including, but not limited to, *Streptomyces natalensis, Streptomyces chattanoogensis, Saccharomyces cerevisiae, Saccharomyces fragilis, Candida utilis,* members of the *Gigartinaceae* and *Soliericeae* families, *Furcellaria fastigiata, Candida guilliermondii, Candida lipolytica,* and (8) organisms that are capable of synthesizing amino acids, including, but not limited to, *E. coli* and other *Enterobacteriaceae* (such as *Klebsiella, Pantoea,* and i strains), *Corynebacterium glutamicum, Brevibacterium* strains, *Bacillus* strains, and *Saccharomyces* strains. Organisms also can be screened for the ability to utilize glutamate rich synthetic or natural polypeptides (e.g., GLURP, the glutamate-rich polypeptide from *Plasmodium falciparum*) as sole nitrogen sources. Such organisms can have the ability to secrete glutamate, allowing them to survive in the presence of high levels of intracellular glutamate, which

may be toxic or may adversely affect cellular osmotic potential. Example 15 indicates that *Pantoea*, specifically *Pantoea stewartii* is capable of monatin production and export.

[0048] Several types of transporter polypeptides can recognize monatin as a substrate such as general amino acid/polyamine exporter polypeptides, dicarboxylic acid exporter polypeptides, auxin secretory polypeptides, and multi-drug resistance polypeptides. In addition, several superfamilies contain transporter polypeptides that can perform monatin efflux. These include 2.A.1 (MFS), 2.A.6 (RND), 2.A.7 (SMR), 2.A.67 (MATE), CAAT (TC 2.A.78), and 2.A.69 (AEC). These superfamilies contain efflux transporter polypeptides that recognize substrates related to auxins (which are structurally similar to monatin derivatives), drugs, antimicrobials, and a wide variety of organic molecules. For instance, the AcrEF polypeptides in *E. coli* (and other RND members such as AcrAB and MexAB) are multiple efflux pumps that expel indoles and many other compounds with hydrophobic domains. In addition, five ABC exporter families contain polypeptides that function to secrete molecules such as polypeptides. These ABC family polypeptides can be used to transport monatin.

[0049] According to some embodiments of the present invention, monatin is produced in a microorganism that can exhibit increased transporter activity as compared to an appropriate control, e.g. as described in examples herein. "Increased transporter activity" is observed by an increase in the amount and/or rate of monatin production or secretion. Without being bound by theory, it is believed that overexpressing pump components, increases the availability of components, translating into an increased likelihood of formation and/or availability of functional transporters systems, and thus increased secretion corresponding to increased production of monatin.

[0050] According to some embodiments, increased transporter activity can be implemented by genetically modifying a microorganism to overexpress one or more types of transporter systems capable of secreting monatin, such as the AcrAB and/or the EmrAB systems.

[0051] According to some embodiments, increased activity can be implemented by genetically modifying a microorganism to overexpress one or more components of a transporter system capable of secreting monatin. For

example, monatin can be produced in a microorganism that is genetically modified to overexpress a channel-forming protein component of a RND family multi-drug transporter, such as overexpressing a channel forming protein (e.g. AcrAB and/or EmrAB) or such as overexpressing individual components of the system, e.g. AcrA and AcrB.

[0052] Production or over-production, of transport systems may be accomplished by various methods. One general method that is known to those of ordinary skill in the art for increasing expression of a gene(s) involved with transport of monatin is to increase the number of gene copies. Increasing the number of gene copies may be achieved by transformingation of an appropriate host microorganism that is capable of monatin transport, with a vector/plasmid carrying the transporter gene(s) of interest, linked to regulatory elements on the vector. This vector with the transporter/transporter component gene could cause the host microorganism to overexpress the respective transporter(s) or components. Another method for increasing the transport system(s) within an organism is using regulatory molecules, such as inducers or repressors.

[0053] Production or over-production of the AcrAB transport system or components thereof may be accomplished by the following methods:

- RamA is a 113-amino-acid regulatory protein belonging to the AraC-XylS transcriptional activator family, in the *Enterobacter aerogenes* ATCC 13048 type strain. Overexpression of RamA increases production of AcrA, a component of the AcrAB-TolC drug efflux pump. Example 17 indicates that overexpression of RamA results in an increase in monatin excretion.
- RamA is also reported to be a transcriptional activator of the marRAB operon and MarA is an activator protein encoded by the marRAB operon. Chollet, R., *et al.*, "RamA is an alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*," *Antimicrob Agents Chemother. 48*:2518-2523, (2004). The marRAB operon is reported to mediate resistance primarily by up-regulating efflux of toxic compounds via the AcrAB-TolC efflux pump.

- It is reported that overexpression of some response regulators of two component signal transduction systems up regulate a number of drug transporter genes including *acrD*, *emrKY*, *mdtABC*, and *mdtEF*. Hirakawa, H., *et al.*, "Indole induces the expression of multidrug transporter genes in *Escherichia coli*," *Molecular Microbiology*, *55*:113-1126, (2005). These are all candidate transporter systems for monatin.
- The baeSR two-component regulatory system activates transcription of the *yegMNOB (mdtABCD)* transporter gene cluster in *Escherichia coli* which is homologous to the AcrAB transport system. Baranova, N., and Nikaido, H., "The baeSR two-component regulatory system activates transcription of the *yegMNOB (mdtABCD)* transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate," *J. Bacteriol. 184*:4168-4176, (2002).
- The BaeSR two-component regulatory system also controls expression of exporter genes conferring drug resistance in *Escherichia coli*. Nagakubo, S. *et al., J. Bacteriol. 184*:4161-4167, (2002); Baranova, N., and Nikaido, H., *J. Bacteriol. 184*:4168-4176, (2002). BaeR overproduction in the absence of the *E.coli* multidrug exporter AcrB confers resistance against a number of toxic substrates including the antibiotic novobiocin. Because AcrAB can transport novobiocin and monatin, this indicates that there are additional transporter(s) activated by BaeR that can transport novobiocin and possibly monatin as well. Nishino K., *et al.*, "Genome-wide analyses of *Escherichia coli* gene expression responsive to the BaeSR two-component regulatory system," *J. Bacteriol. 187*:1763-1772, (March 2005). Example 19 indicates that overexpression of BaeR results in increased monatin excretion.
- Mutations in *marR* result in increased expression of acrAB genes. (Ma, D., *et al.*, "Genes *acrA* and *acrB* encode a stress-induced efflux system of *E.coli*," *Mol. Microbiol. 16*:45-55, (1995)) and in strains carrying multicopy plasmids expressing *marA*. Miller, P.F., and Sulavik, M.C, "Overlaps and parallels in the regulation of intrinsic

multiple-antibiotic resistance in *Escherichia coli*," *Mol. Microbiol. 21*:441-448 (1996). Thus, overexpression of the *marA* gene or an inactive *marR* gene result in an increase in the AcrAB transport system.

- MarA, SoxS, and SidA (members of the XylS/AraC family of transcriptional regulators) are global regulators and activate the expression of AcrAB transport system. AcrAB and three other *E.coli* genes involved with multi drug resistance (and also candidates for monatin transport) *tolC, acrEF* and *acrD* are also activated by SdiA. Baranova, N., and Nikaido, H., "The baeSR two-component regulatory system activates transcription of the *yegMNOB (mdtABCD)* transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate," *J Bacteriol. 184*:4168-4176, (2002). Example 18 indicates that overexpression of MarA results in increased monatin excretion.

[0054] According to some embodiments, increased activity of AcrEF, a pump which is highly homologous to AcrAB and therefore is also expected to secrete monatin could be accomplished by the following modification:

- The presence of insertion elements IS1 and IS10 elements containing putative promoter sequences result in an 8- to 10-fold increase in expression of acrF expression in these insertional mutants, thus, increasing AcrEF activity and possibly monatin transport in a host that is capable of monatin production. Olliver A., *et al.*, "Overexpression of the multidrug efflux operon *acrEF* by insertional activation with IS1 or IS10 elements in *Salmonella enterica* serovar *typhimurium* DT204 *acrB* mutants selected with fluoroquinolones," *Antimicrob Agents Chemother. 49*:289-301 (January 2005). In a different case an *E.coli* strain in which the *acrEF* operon had IS1 or IS2 integrated upstream produced high levels of AcrE and AcrF proteins. Kobayashi K., *et al.* "Suppression of hypersensitivity of *Escherichia coli acrB* mutant to organic solvents by integrational activation of the *acrEF* operon with the IS1 or IS2 element, *J Bacteriol. 183*:2646-2653 (2001).

The examples listed above describe some ways to overexpress the AcrEF transport system which could transport monatin because it is highly homologous to the AcrAB transport system;

- EnvR (formerly envCD) is a transcriptional repressor of the *acrEF* operon. Therefore a non-functional EnvR regulator might increase *acrEF* transcription. Increasing *acrEF* transcription results in overexpressing the AcrEF transport system which could transport monatin because it is highly homologous to the AcrAB transport system.

[0055] According to some embodiments, increased transporter activity can be implemented by: overexpressing an outer membrane factor component of a RND family multi-drug transporter, such as TolC; by overexpressing other transport system components which cooperate with TolC to form functional transporters in organisms whose wild-type form expresses TolC; or combinations thereof. The transport system components that cooperate with TolcC may be non-genetically engineered. Any method known in the art for screening microorganisms for the existence of functional TolC could be used. *See*, for example, Werner, J., *et al.*, "Assembly of TolC, a structurally unique and multifunctional outer membrane protein of *Escherichia coli* K-12," *J. Bact. 185*:6540-6547 (2003). In addition, Example 5 describes methods that can be used to clone and overexpress TolC, however any method known in the art can be used. Example 6 demonstrates that overexpression of TolC increases secretion of monatin. Because TolC functions in conjunction with several different efflux pumps, overexpression of TolC could lead to even greater increased transporter activity than would occur by overexpressing other components of transporters which are more discriminating.

[0056] TolC-dependent machineries present ubiquitous exit routes for virulence proteins and antibacterial drugs. Koronakis, V., "TolC--the bacterial exit duct for proteins and drugs," *FEBS Lett. 555*:66-71, (2003). Based on the results from Example 6, any host strain capable of producing monatin and that has efflux pumps/transport systems (for example AcrAB and EmrAB) that work in conjunction with TolC might show an increase in monatin transport

due to an increase in TolC channel presence. Non-limiting examples of such other transporter systems include:

- A four-component type I secretion system (TISS) encoded by *rtxB, rtxD, rtxE,* and *tolC* in *Vibrio cholerae.* Boardman, B.K., and Satchell, K.J., "*Vibrio cholerae* strains with mutations in an atypical type I secretion system accumulate RTX toxin intracellularly," *J Bacteriol. 186*:8137-8143, (2004).
- EmrKY and YhiUV are three component efflux pumps requiring TolC for their activity. Fralick, J.A., "Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *E.coli.*," *J. Bact. 178*:5803-5805, (1996); Nishino, K. and Yamaguchi, A., "EvgA of the two-component signal transduction system modulates production of the YhiUV multidrug transport in *Escherichia coli,*" *J. Bact. 184*:2319-2323, (2002)

[0057] Similarly, TolC homologs might also cooperate with the AcrAB system to provide improvements in the production rate or amount of monatin. Overall comparisons between the TolC family members demonstrate that the characteristic structural elements are conserved. This strongly indicates that all homologs fold similarly and have comparable properties. Conservation of the key structural amino acids among TolC homologs establishes a common mechanism for the export and efflux systems that involve the TolC family of proteins. It is reported that one can conclude that the core functions of the channel-tunnel are common throughout Gram-negative bacteria. Andersen, C., *et al.*, "Chunnel vision. Export and efflux through bacterial channel-tunnels," *EMBO Rep. 1*:313-8, (2000). Non-limiting examples of homologs that may also have utility in the secretion of monatin include:

- Nearly a hundred homologs identified in over 30 bacterial species consistent with reports that TolC family members are widespread among Gram-negative bacteria. Dinh, T., *et al.*, "A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria," *J Bacteriol. 176*:3825-3831, (1994); Andersen, C., *et al.*, "Chunnel vision. Export

and efflux through bacterial channel-tunnels," *EMBO Rep. 1*:313-318, (2000).

- The *E.coli* genome encodes three *tolC* homologs and approximately 30 inner membrane translocases of the ABC, MFS (EmrAB) and RND (AcrAB) families.
- *Pseudomonas aeruginosa* has four major efflux (Mex) systems containing and RNA proton antiporter and one of three TolC homologs, OprM, OprJ and Opr N. These observations are consistent with the fact that bacteria are reported to have several TolC homologs acting in parallel with a number of efflux pumps with broad and sometimes overlapping specificities. Koronakis V., *et al.*, "Structure and function of TolC: the bacterial exit duct for proteins and drugs," *Annu Rev Biochem. 73*:467-489, (2004).
- Some TolC homologs involved in drug efflux (and that could be potentially involved in monatin efflux) are: FusA, OprA, OpcM, NodT3, NodT2, NodT1, SmeC, SrpC, TtgC, MtrE. Andersen C., *et al.*, "Chunnel vision. Export and efflux through bacterial channel-tunnels," *EMBO Rep. 1*:313-318 (2000).

[0058] Activity of TolC may also be increased to achieve enhanced secretion of monatin. Non-limiting examples of potential methods of increasing TolC activity include:

- TolC is upregulated by MarA. The multiple antibiotic resistance (*mar*) locus of *Escherichia coli* and *Salmonella* is perhaps the best described system involved in this type of resistance which is induced by MarA, the activator protein encoded by the *marRAB* locus. The mar locus is reported to mediate resistance primarily by up-regulating efflux of some antibiotics, disinfectants and organic solvents via the AcrAB-TolC efflux pump. Randall, L.P., and Woodward, M.J., "The multiple antibiotic resistance (mar) locus and its significance," *Res Vet Sci. 72*:87-93, (2002).
- The TolC level is regulated positively by Rob, or SoxS, in addition to MarA. A possible *mar-rob-sox* box sequence is present upstream of the *tolC* gene. These findings suggest that *tolC* is a member of the mar-

sox regulon responsive to stress conditions. *Escherichia coli* mutants with improved organic solvent tolerance levels showed high levels of outer membrane protein TolC and inner membrane protein AcrA. Aono, R., *et al.*, "Involvement of outer membrane protein TolC, a possible member of the *mar-sox* regulon, in maintenance and improvement of organic solvent tolerance of *Escherichia coli* K-12," *J Bacteriol. 180*:938-944 (1998).

- RamA is a regulatory protein that enhances *marA* transcription which in turn causes *tolC* overexpression. Chollet, R., *et al.*, "RamA is an alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*," *Antimicrob Agents Chemother. 48*:2518-2523, (2004). Example 17 indicates that overexpression of RamA results in increased monatin excretion.

[0059] According to some embodiments, increased transporter activity is implemented by exposing a microorganism expressing a pump capable of secreting monatin to an inducing compound (i.e. a compound that triggers expression of a transporter system or component of a transporter system). For example, a microorganism expressing the AcrAB system can be exposed to sodium decanoate or salicylate. Example 1 demonstrates that sodium decanoate can be used to induce the AcrAB pump to secrete monatin. As another example, a microorganism expressing the EmrAB system may be exposed to carbonyl 2-chlorophenylhydrazone ("CCCP") in its growth medium. Example 2 demonstrates that CCCP can induce increased monatin transport. Example 14 demonstrates that salicylate can induce increased monatin transport.

[0060] Other potential inducers of AcrAB include, but are not limited to:

- Phytoalexin is an inducer of the AcrAB transport system. Burse, A., *et al.*, "The phytoalexin-inducible multidrug efflux pump AcrAB contributes to virulence in the fire blight pathogen, *Erwinia amylovora*," *Mol Plant Microbe Interact. 17*:43-54, (2004).
- Limitation of nutrients such as glucose, iron or nitrogen act as inducing conditions for overexpression of AcrAB transport system. It is reported that *acrAB* is regulated as a function of the growth rate of *E.*

coli during growth in batch and chemostat culture. In chemostat culture, expression of *acrAB* is inversely related to growth rate irrespective of the limiting nutrient. The level of expression of *acrAB* is greater under glucose limitation compared with either iron or nitrogen limitation. Slow growth rate regulation of *acrAB* transcription does not require the presence of the stationary-phase sigma factor. A putative gearbox consensus sequence was identified at the -10 region of the acrAB promoter. Rand, J.D., *et al.*, "Increased expression of the multidrug efflux genes acrAB occurs during slow growth/stationary phase of *Escherichia coli*," *FEMS Microbiol Lett. 207*:91-95 (2002).

- RobA is a member of the XylS/AraC subfamily of DNA binding proteins and activates the AcrAB transport system. When *robA* is overexpressed, it induces multiple antibiotic resistance in *Escherichia coli*. It is reported that the multiple antibiotic resistance induced by the overexpression of RobA largely depends on the activation of the AcrAB efflux, as well as the activation of *micF*. Tanaka, T., *et al.*, "RobA-induced multiple antibiotic resistance largely depends on the activation of the AcrAB efflux," *Microbiol Immunol. 41*:697-702, (1997). Example 16 indicates that overexpression of RobA results in increased monatin excretion.

- *cysH*, *icdA* (isocitrate dehydrogenase), *metE*, or *purB* (adenylosuccinate lyase) mutations cause an activation of the AcrAB transport system. While screening mutants of *E.coli* and other bacteria on nutrient plates with low levels of nalidixic acid, it is reported that resistance results from mutations at different genetic loci. About 10% of the nalidixic acid-resistant (Nal^r) mutants in a transposition-induced library exhibit a growth factor requirement as the result of *cysH*, *icdA* (isocitrate dehydrogenase), *metE*, or *purB* (adenylosuccinate lyase) mutation. Resistance in all of these mutants requires a functional AcrAB-TolC efflux pump. Transcription of *acrAB* increases in each type of Nal(r) mutant. In the *icdA* and *purB* mutants, each of the known signaling pathways could be used to activate the AcrAB-TolC pump. The metabolites that accumulate upstream of the blocks caused

by the mutations could increase the levels of the AcrAB-TolC pump, thereby removing nalidixic acid from the organism. Helling, *et al.*, "Toxic waste disposal in *Escherichia coli*," *J Bacteriol. 184*:3699-3703, (2002). Each of the mutations above could be generated in a host strain capable of monatin production to increase the monatin transported via the AcrAB transporter. Example 23 illustrates deletion of the *cysH* gene results in increased monatin production.

- Salicylate induces the AcrAB-TolC efflux pump by at least two mechanisms, one of which involves Mar. Cohen, S.P. *et al.*, "Salicylate induction of the antibiotic resistance in *Escherichia coli*: activation of the *mar* operon and *mar*-independent pathway," *J. Bacteriol. 175*:7856-7862, (1993).

- Several global stress signals such as 4% ethanol, fatty acids like decanoate, or media of high osmolarity (0.4M NaCl) enhance the expression of AcrAB transport system. Thus one could use environmental conditions to indirectly affect expression of the AcrAB transport system. Ma D, *et al.* "Efflux pumps and drug resistance in gram-negative bacteria," *Trends Microbiol. 2*:489-493 (1994).

- One general method that is known to those skilled in the art for increasing the expression of a gene(s) involved with transport of monatin is to increase the number of gene copies. This could be achieved by transformation of an appropriate host microorganism that is capable of monatin transport, with a vector/plasmid carrying the transporter gene(s) of interest, linked to regulatory elements on the vector. This vector with the transporter/transporter component gene could cause the host microorganism to overexpress the respective transporter(s) or components.

[0061] Another embodiment in accordance with the invention comprises producing monatin in a glutamate auxotroph, which is an organism that has lost the ability to synthesize glutamate as the result of mutational changes. Without being bound by theory, the inventors hypothesized that transporters useful for secreting glutamate may also secrete monatin. A glutamate

auxotroph would likely still have the glutamate transporters, but because there would be more carbon available in the cell to make monatin (because it is not being consumed to make glutamate) and/or because there would not be competition for the glutamate transporters from glutamate, glutamate auxotrophs may be suitable for producing and secreting monatin.

[0062] One method for preparing a glutamate auxotroph is provided in Eikmanns, B.J., *et al.*, "Cloning, sequence analysis, and inactivation of the *Corynebacterium glutamicum icd* gene encoding isocitrate dehydrogenase and biochemical characterization of the enzyme," *J Bacteriol.*, *177*:774-782, (1995). However, any method known in the art can be used.

[0063] Example 8 provides an example of a host strain, *Corynebacterium glutamacium* ATCC strain 13032, which has been mutated to become a glutamate auxotroph, and demonstrates increased excretion of monatin in this strain. Example 9 provides an example of a strain, *E. coli* glutamate auxotroph (*icd*A deficient), which is expected to increase monatin excretion potential.

[0064] It is contemplated that a glutamate auxotroph adapted to produce monatin could be combined with one or more other methods to provide secretion of monatin to further increase monatin transport. Glutamate auxotrophs (with an inactivated *icd* gene) transformed with the genes required for monatin production, combined with other treatments and cell modifications predicted or shown to increase monatin production and/or transport could show further increases in monatin production/transport. For example, the glutamate auxotroph may be exposed to inducers for the AcrAB and/or the EmrAB transporters; or the glutamate auxotroph may be engineered to overexpress transporters or transporter components such as TolC; or the fermentation medium components could be modified to include detergents such as Tween 20/40/60 and/or ampicillin (10 μg/mL); or combinations thereof.

[0065] Another embodiment in accordance with the invention for secreting monatin involves genetically modifying a microorganism capable of translocating glutamate or oxoglutarate in exchange for malate to have the ability to produce monatin. A related embodiment involves producing

monatin in such an organism wherein that organism has also been genetically modified to be a glutamate auxotroph. Example 10 illustrates use of a microorganism capable of translocating gluatamate for malate in the production of monatin.

[0066] Without being bound by theory, monatin has a glutamate backbone or can be considered to be a 4-substituted glutamate derivative. Monatin may therefore be transported out of a bacterial cell by transporters that translocate glutamate in exchange for substrates like malate. The glutamate/malate transporter in *Arabidopsis* plastids, encoded by the DiT2 gene, translocates glutamate and malate in antiport manner. Renne, P., *et al.*, "The *Arabidopsis* mutant *dct* is deficient in the plastidic glutamate/malate translocator DiT2," *Plant J. 35*:316-331, (2003); Taniguchi, M., *et al.*, "Identifying and Characterizing Plastidic 2-Oxoglutarate/Malate and Dicarboxylate Transporters in *Arabidopsis thaliana*," *Plant and Cell Physiology 43*:706-717, (2002). The glutamate/malate transporter family is homologous with the 2-oxoglutarate / malate transporter in spinach chloroplasts which is related to the CitT transporter in *E.coli* that is believed to be an antiporter for citrate and succinate. Pos, K.M., *et al.*, "The Escherichia coli Citrate Carrier CitT: a Member of a Novel Eubacterial Transporter Family Related to the 2-Oxoglutarate/Malate Translocator from Spinach Chloroplasts," *Journal of Bacteriology 180*:4160-4165, (1998). There is a possibility that this transporter could permit a host to take up malate while excreting glutamate or monatin into the supernatant. Another possibility for malate functioning to increase monatin transport could be due to the role that malate might play as an alternative carbon source affecting growth rate and carbon distribution to metabolic pathways differently than with glucose. Malate can also be converted to pyruvate internally by malic enzymes encoded by 2 genes (Fischer, E., and Sauer, U., "Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS," *Eur. J. Biochem. 270*:880-891, (2003)), and, because pyruvate is one of the precursors to monatin, a greater availability of pyruvate may result in increased monatin production and consequently increased monatin secretion (see other references to induction of transporters in microorganisms to get rid of accumulated metabolites). Growth of *E. coli* on malate as the primary carbon source

resulted in increases in monatin excretion into the medium, such as the culture medium.

[0067] In another embodiment according to the invention, the amount of monatin produced may be affected by temperature and/or treatment with one or more additional compounds, such as those that perturb the cell membrane (ampicillin and Tween). Additionally, the efflux of monatin from the microorganism may be increased by selecting an optimal temperature and/or by treating the microorganism with one or more additional compounds, such as those that perturb the cell membrane (ampicillin and Tween). Examples of suitable compounds for these effects include ethambutol, ampicillin, Tween and/or biotin. For example, Example 20 indicates that an increase in temperature, as well as an increase in the amount of sodium pyruvate provided to *Corneybacterium glutamicum* cells resulted in increased monatin efflux. Additionally, Example 21 indicates that treatment with ampicillin alone, or in combination with biotin, resulted in increased efflux of monatin. Further, Example 22 demonstrates that treatment with ethambutol, alone or in combination with Tween and ampicillin, had a positive impact on monatin efflux in *Corynebacterium*.

[0068] Another embodiment according to the invention includes producing monatin in a microorganism which is selected because it does not express certain transporters, or is engineered so that it does not express certain transporters. Example 11 demonstrates that the absence of certain transporters—the four Putative Efflux Transporters identified as YhcP (AaeB), YccS, YjcQ and YhfK, lead to increased production of monatin. Without being bound by theory, it is believed that certain pumps may also transport intermediates formed along the pathway for production of monatin, and the excretion of these intermediates would result in slower or decreased production of monatin. Consequently, absence of these pumps could lead to faster or increased production of monatin.

[0069] Another embodiment according to the invention includes producing monatin in a microorganism which is engineered to have a modified cell envelope, for example a microorganism that is engineered to be deficient or depressed in mycoloic acids. Without being bound by theory, it is believed that such an approach may lead to increased monatin efflux due to a weakened

outer permeability barrier. More specifically, mycolic acids, the major lipid constituents of the cell envelope of the *Corynebacterineae*, are found covalently linked to the cell-wall arabinogalactan or esterifying trehalose and glycerol. Mycolic-acid-containing components are believed to play a crucial role in the structure and function of this cell envelope, primarily because they are organized with other lipids to form an outer permeability barrier with an extremely low fluidity that confers an exceptionally low permeability upon these bacteria; this may explain the intrinsic resistance of mycobacteria to many antibiotics. Kacem, R., *et al.*, "Importance of mycoloyltransferases on the physiology of *Corynebacterium glutamicum*," *Microbiology 150*:73-84, (2004).

[0070] The monatin produced by the microorganism may be collected from the medium after it has been secreted. Additionally, the monatin produced by the microorganism may be isolated from the medium after it has been secreted. Separation methods are known to those in the art for the isolation of organic acids from fermentation media, which typically rely on chromatography methods and/or extractions. Monatin is similar to glutamic acid. Many methods are known in the art for purification of glutamic acid from fermentation broths. A description of the isolation of monatin from a complex biological medium has been previously described (see WO03091396 Example 6). One example of a method that may be used to collect and/or isolate the monatin from the medium is to use strong cation exchange chromatography at a low pH, such as the AG50WX-8 resin (H form) from Bio-Rad. In this method, the amino group of the compound, monatin, is charged and is bound to the resin. Any contamining organic acids are not bound to the resin and flow through the resin at low pH. The amino acids may then be separated from each other (such as separating tryptophan from alanine from monatin) using anion exchange chromatography, such as a DEAE resin, at a neutral pH.

[0071] The following Examples are intended to assist one of ordinary skill in making, using, and/or understanding the present invention. These Examples are not intended in any way to limit the scope of the disclosure. For example, the monatin used in the Examples is predominately S,S monatin. However, the specificity of the transporters in the Examples is not expected to be based

on chirality of the transported molecules. Therefore, the systems demonstrated to transport S,S monatin should be effective in transporting all four stereoismers of monatin.

EXAMPLES

EXAMPLE 1

Induction of the AcrAB Efflux Pump Increased Monatin Transport

[0072] The AcrAB TolC system of *Escherichia coli* is a multidrug efflux pump composed of a cytoplasmic membrane component/proton antiporter AcrB and a periplasmic accessory protein AcrA. Accession numbers for AcrA and AcrB are AcrA (protein, NP_414996, DNA, NC_000913) and AcrB (protein, NP_414995, DNA, NC_000913). The cell uses this system to pump out a wide variety of antimicrobial compounds, including antibiotics, detergents, dyes, and organic solvents directly into the medium through TolC, an outer membrane channel. The AcrAB genes are inducible by addition of sodium decanoate. Zgurskaya, H.I., and Nikaido, H., *Proc Natl Acad Sci USA 96*:7190-7195, (1999).

[0073] A preliminary study with *E.coli* BL21 DE3 was done that determined that 2.5 mM sodium decanoate addition to the medium resulted in tolerance to 80 – 160 µg / ml novobiocin. This was taken as evidence that *acrAB* genes are induced with sodium decanoate addition and conferred resistance to novobiocin. Rosenberg, E.Y., *et al.*, *Molecular Microbiol. 48*:1609-1619, (2003).

[0074] The microbial strain used for the experiment was *E.coli* BL21 (DE3):: *aspC* / *proA* /pET32 (WO 03091396). The symbol ::, as is known in the art, stands for "transformed." Example 12 provides a non-limiting exemplary method for transforming a microorganism. For an inoculum, the *E.coli* strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 100 µg/mL ampicillin. For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents

were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt, F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746 (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ –$6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume. 2-5 v/v % of *E.coli* inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 100 µg/mL ampicillin. Flasks were incubated at 37 °C with agitation at 250 rpm up to induction. At 0.6 OD_{600nm}, induction of the monatin operon genes (*aspC* and *proA*) on the pET32 vector was initiated using 0.5 mM IPTG. 0.5 mM pyridoxine hydrochloride, and 0.2 mL of Balch's vitamins (Balch, W.E., *et al.*, "Methanogens: reevaluation of a unique biological group," *Microbiol. Rev. 43*:260-296, (1979)) were added at induction and the incubation temperature was lowered to 30 °C following induction. Additions of 1 g L-tryptophan, 5 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3.5 hours following induction. Some treatments included 2.5 mM sodium decanoate addition at 3.5 hours following induction. Samples for monatin analysis and dry cell weight determination were taken at 24 and 30 hours. Monatin analysis was done as described in Example 13.

Table 1.1

Monatin per dry cell weight effluxed by *E.coli*

		Monatin/dcw. at run hour:	
Trtmt. no.	**Treatment**	**24**	**30**
1	2.5 mM Na decanoate	17.0	17.4
2	no decanoate	1.3	1.3
3	2.5 mM Na decanoate	14.7	14.3
4	no decanoate	1.7	1.6
5	2.5 mM Na decanoate	12.0	13.8
6	no decanoate	1.8	1.8

Monatin / dcw = mg monatin / g dcw

Table 1.2

Average monatin per dry cell weight effluxed by *E.coli*

	Monatin/dcw. at run hour:	
Treatment	**24**	**30**
2.5 mM Na decanoate	14.6	15.2
no decanoate	1.58	1.57

Monatin / dcw = mg monatin / g dcw

n=3

[0075] Greater than nine fold increase in monatin secreted/dry cell weight ("dcw") (14.6 to 1.58 or 15.2 to 1.57 mg monatin / g dcw) was observed by treating *E.coli* BL21 (DE3):: *aspC* / *proA* /pET32 with 2.5 mM sodium decanoate which induces the AcrAB efflux system. Monatin excretion can therefore be increased by turning on or up-regulating expression of the AcrAB efflux system. Transporter system homologs of the AcrAB transport system, when exposed to appropriate inducers, might also increase monatin transport.

EXAMPLE 2

Induction of the EmrAB Efflux Pump Increased Monatin Transport - *E.coli and C. glutamicum.*

[0076] A multidrug efflux pump is encoded by the *emrB* gene, the EmrB efflux pump (GenBank Accession Number NP_417171, DNA NC_000913). Lomovskaya, O., and Lewis, K, "*emr,* an *E.coli* locus for multidrug resistance," *Proc. Natl. Acad. Sci. USA 89*:8938-8942, (1992). The *emrB* gene can be upregulated by addition of the inducer carbonyl cyanide 3-chlorophenylhydrazone ("CCCP") to the growth/fermentation medium. Lomovskaya O., *et al.*, "Differential regulation of the *mcb* and *emr* operons of *E.coli*: Role of mcb in multidrug resistance," *Antimicrob Agents Chemother. 40*:1050-1052, (1996). This example shows increased monatin efflux as a result of CCCP treatment.

[0077] The strains used for the experiment included *E.coli* MG1655 :: *aspC /proA* / pProNde and *E.coli* BL21 (DE3):: *aspC /proA* / pET30. For inoculum, the *E.coli* strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 50 μg/mL kanamycin.

[0078] For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman, *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt, F:C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746 (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ $-6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0079] For treatments, 2-5 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 50 μg/mL kanamycin. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then, 30°C following induction. At 0.5-0.6 OD_{600nm} , induction of the plasmid genes was initiated. At induction, 0.5 mM IPTG, 0.5% arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mL of Balch's vitamins were added. Additions of 1 g L-tryptophan, 5 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP"), 10 μg/ml ampicillin and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3.5 hours following induction. Some treatments included 10 μM carbonyl cyanide 3-chlorophenylhydrazone ("CCCP") addition to initial medium. One treatment included an additional 10 μM CCCP dose at 3.5 hours following induction. CCCP induces the EmrB efflux system. Samples for monatin analysis and dry cell weight determination were taken at 10, 15.5, 25.6 and 31 hours. Monatin analysis was preformed as described in Example 13.

Table 2.1

Monatin per dry cell weight effluxed by *E.coli* MG1655 and *E.coli* BL21 (DE3)

Strain	**Treatment**	***Monatin / dry cell weight at run hour:**			
		10	**15.5**	**25.6**	**31**
E.coli MG1655:: *aspCproA* pProNde	control (no CCCP**)		0.5	3.3	6.2
E.coli MG1655:: *aspCproA* pProNde	10 μM CCCP		0.7	6.7	12.4
E.coli BL21 (DE3) :: *aspCproA* pET30	control (no CCCP)	3.9	9.6	19.1	18.1
E.coli BL21 (DE3) :: *aspCproA* pET30	10 μM CCCP	6.4	22.5	34.3	33.0
E.coli BL21 (DE3) :: *aspCproA* pET30	10 μM CCCP + 10 μM CCCP	16.2	38.0	52.1	64.2

*Monatin / dcw is in mg /g

**CCCP is carbonyl cyanide 3-chlorophenylhydrazone

[0080] With the *E.*coli BL21 (DE3) :: *aspC proA* pET30 strain, an increase of greater than 1.8 or 3.5-fold monatin /dcw (33.0 / 18.1 or 64.2 / 18.1) was

obtained at 31 hours by treating shake flasks with one (10 μM) or two additions (20 μM) of CCCP. With the *E.coli* MG1655::*aspC proA* pProNde strain, a 2-fold increase in monatin /dcw was observed at 31 hours. Monatin efflux can therefore be increased at least two fold by turning on or upregulating expression of the EmrAB efflux pump. Monatin efflux can be further increased by combination with other treatments shown to increase monatin transport.

EXAMPLE 3

Knockout of *E. coli emrB* and *acrAB* Genes to Test Impact on Monatin Transport by the EmrAB and AcrAB Transporters Respectively

[0081] Primers were designed to create the desired knockout product by PCR from template pKD3 as described. Datsenko K.A., and Wanner, B.L., "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products," *Proceed. Natl. Acad. Sci. USA, 97*:6640-6645, (2000).

emrB knockout primer sequences:

E. coli EmrBF (5'AAGCTAACGCTGGCTAATCCAGAGGTGCGTGTGATGGTGTAGGC TGGAGCTGCTTC - 3');

E. coli EmrBR (5' - AAAGCCAGTTCAAATGAACTGGCTTAGTTGTACTTACATATGAATA TCCTCCTTA - 3');

acrAB knockout primer sequences:

E. coli AcrAF (5' - GACCAATTTGAAATCGGACACTCGAGGTTTACATATGAGTGTAGGC TGGAGCTGCTTC - 3'); *E. coli* AcrBR (5' - CTTACGCGGCCTTAGTGATTACACGTTGTATCAATGATGCATATGA ATATCCTCCTTA - 3')

[0082] The PCR products for deletion of *emrB* and *acrAB* genes were amplified using the following PCR protocol. In a 100 µL reaction, 100 ng of template (pKD3) (Datsenko, K.A., and Wanner, B.L., "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products," *Proceed. Natl. Acad. Sci. USA 97*:6640-6645, (2000)), 0.4 µM of each primer, 0.4 mM each dNTP, 5.6 U Expand High Fidelity™ Polymerase (Roche, Indianapolis, IN), 1.0 U Pfu polymerase (Stratagene, La Jolla, CA) and 1X Expand™ buffer with Mg were used. The thermocycler program used included a hot start at 94 °C for 3 minutes, 8 repetitions of the following steps: 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1 minute 30 seconds, followed by 22 repetitions of the following steps: 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 1 minute 30 seconds. After the 22 repetitions the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a product of 1.1-Kb for both *emrB* and *acrAB* knockout primer pairs.

[0083] The PCR products were gel purified from 0.8% TAE-agarose gels using the Qiagen gel extraction kit (Valencia, CA). The PCR products were quantified using a SmartSpec 3000™ spectrophotometer.

[0084] The gel-purified PCR products were used to transform *E. coli* strain BW25113/pKD46. Datsenko K.A., and Wanner, B.L., "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products," *Proceed. Natl. Acad. Sci. USA, 97*:6640-6645, (2000). 1 µL of each product was added to 40 µl of cells, which were transformed by electroporation using the BioRad Gene Pulsar II under the following conditions: 2.5 kV, 25 µF, 200 ohm in a 0.2 cm cuvette. The cells were allowed to recover in 1 mL of SOC for 4 hours at 37 °C with shaking at 225 rpm, and then placed at room temperature (no shaking) overnight. Cells were plated on LB plates containing chloramphenicol (10 µg/mL) and incubated at 37 °C overnight. Choloramphenicol-resistant transformants were single-colony purified on non-selective LB medium (grown at 42 °C), and single colonies were tested for retention of chloramphenicol resistance and loss of ampicillin resistance (indicating curing of pKD46). Confirmation of the correct deletion of the

emrB or *acrAB* genes was carried out by colony PCR using primers upstream and downstream of the deletion locus:

EmrBupstreamF: 5' - GTATCGGTCAGCCGGTCACT - 3'

EmrBdownstreamR: 5'- TGTTCGATCTGCGCTTCTGC - 3'

AcrAupstreamF: 5'- TAATCGACGCCGTTCTTCTG - 3'

AcrBdownstreamR: 5' – GCGGTTGAACTAACGGACAC - 3'

[0085] For deletion of *emrB*, a truncated 1.9 kb PCR product was observed as compared to the wild-type 2.4 kb product using primers EmrBupstream F and EmrBdownstreamR. For deletion of *acrAB*, a truncated 1.9 Kb product was observed as compared to the wild-type 5.679 kb product using primers AcrAupstreamF and AcrBdownstreamR.

[0086] **Lysate production**: P1 phage lysates were made for the BW25113ΔEmrB and BW25113ΔAcrAB strains, to allow transfer of the knockouts into the *E. coli* BL21DE3 or *E. coli* MG1655 production hosts respectively. Donor strains were grown overnight in LB medium containing 10 μg/mL chloramphenicol. The cultures were used to inoculate fresh LB medium containing 5 mM $CaCl_2$ using a 1:10 dilution and were incubated for 70 minutes at 37 °C. One mL of each culture was incubated with 3 μL or 5 μL of a phage stock (ATCC 25404-B1) at 37°C for 20 minutes. The phage/culture was then mixed with 4 mL of soft agar containing 5 mM $CaCl_2$ and overlaid on LB medium. Control experiments were set up using no phage. Plates were incubated at 37 °C, right-side up for 5 hours, after which confluent lysis was observed for all plates containing phage; control plates had cell lawns as expected. Plates were incubated overnight at 37 °C, after which phage-resistant colonies were observed on experimental plates as expected. The soft agar from each plate was scraped into a centrifuge tube using a sterile disposable loop. Two mL of LB was used to rinse the plate, and the rinse was combined with the soft agar in the centrifuge tube. Five drops of chloroform were added to the tubes, which were gently mixed and incubated at room temperature for 20 minutes. The mixtures were centrifuged at 10,000 g for 10

minutes and the supernatants filtered with a 0.2 μm syringe filter to obtain phage lysates. All phage lysates were stored at 4°C.

[0087] **Transduction into production hosts:** The *emrB* knockout was transferred to strain *E. coli* BL21DE3 and the *acrAB* knockout was transferred to strain *E. coli* MG1655 by P1 transduction to generate strains BL21DE3Δ*emrB* and MG1655Δ*acrAB* respectively. BL21DE3Δ*emrB* and MG1655Δ*acrAB* were grown overnight in LB medium containing 10 μg/mL chloramphenicol. Cultures were used to inoculate 5 mL of fresh LB medium supplemented with 5 mM $CaCl_2$ using a 1:10 dilution. The subcultures were incubated for 60 minutes at 37 °C. The cultures was centrifuged, resuspended in 500 μL of MC buffer (0.1 M $MgSO_4$, 5 mM $CaCl_2$), and incubated at room temperature for 20 minutes. Various dilutions of the donor lysate (1:100 to 1X in MC buffer) were added in equal volume to 100 μL of culture. The mixtures were incubated for 20 minutes at 37 °C, after which 200 μL of citrate buffer (0.1 M citric acid and 220 mM NaOH adjusted to pH 5.5) and one mL of LB were added to each tube. The cultures were incubated at 37 °C for one hour with agitation at 200 rpm, followed by centrifugation to obtain a cell pellet. Cell pellets were resuspended in 100 μL of citrate buffer and plated on LB medium containing 10 μg/mL chloramphenicol.

[0088] Single chloramphenicol-resistant colonies were purified by restreaking on appropriate selective media and single colonies were tested by PCR as previously described for the BW25113 knockout strains, to verify the presence of the *emrB* and *acrAB* knockouts. The effects of the *emrB* and *acrAB* knockouts on the EmrAB and AcrAB transporter systems respectively were determined by assessing the phenotype of the transport mutants using appropriate antibiotics and comparison to wild type control microorganisms as shown in Table 3.1 and Table 3.2 below.

Table 3.1

		Treatment	Optical Density @ 600 nM	
Strain	**Strain type**	**CCCP* (μM)**	**6 hours**	**24 hours**
E.coli BL21DE3ΔemrB:: *aspCproA*pET30	*emrB* knockout mutant	20	0.024	0.014
E.coli BL21DE3 :: *aspCproA*pet30	wild type control	20	0.034	1.113
E.coli BL21DE3ΔemrB:: *aspCproA*pET30	*emrB* knockout mutant	0	0.787	1.452
E.coli BL21DE3 :: *aspCproA*pET30	wild type control	0	1.021	1.597

*Carbonyl cyanide 3-chlorophenylhydrazone

[0089] Thus it was confirmed that the EmrAB transporter system is responsible for the efflux of CCCP, based on the observations in Table 3.1. In the absence of CCCP (0 μM), similar growth is observed at 24 hours for both wild type and Δ*emrB E.coli* strains, transformed with the monatin operon (*aspC, proA*) on the pET30 vector. However upon the addition of 20 μM CCCP, the deletion strain, *E.coli* BL21DE3ΔemrB:: *aspCproA*pET30, shows an eighty fold/ninety nine percent decrease in growth, presumably due to an inability to efflux the toxic molecule CCCP. These data confirm the major role of the EmrAB system in transporting CCCP.

Table 3.2

AcrAB k.o. validation	Treatment		
Strain	sodium decanoate (mM)	novobiocin (ppm)	OD 600nm at 19 hours
E.coli MG1655 ΔAcrAB 6-16 :: *aspCproA*proNde	0	0	1.436
E.coli MG1655 ΔAcrAB 6-16 :: *aspCproA*proNde	0	40	0.007
E.coli MG1655 ΔAcrAB 6-16 :: *aspCproA*proNde	0	80	0.006
E.coli MG1655 :: *aspCproA*pProNde (control)	0	0	1.556
E.coli MG1655 :: *aspCproA*pProNde (control)	0	40	1.407
E.coli MG1655 :: *aspCproA*pProNde (control)	0	80	0.793
E.coli MG1655 ΔAcrAB 6-16 :: *aspCproA*proNde	2.5	0	0.885
E.coli MG1655 ΔAcrAB 6-16 :: *aspCproA*proNde	2.5	40	0.008
E.coli MG1655 ΔAcrAB 6-16 :: *aspCproA*proNde	2.5	80	0.008
E.coli MG1655 :: *aspCproA*pProNde (control)	2.5	0	1.396
E.coli MG1655 :: *aspCproA*pProNde (control)	2.5	40	1.395
E.coli MG1655 :: *aspCproA*pProNde (control)	2.5	80	1.374

[0090] Thus, it was confirmed that the AcrAB transporter system is responsible for the efflux of novobiocin, and that this system is induced by sodium decanoate, based on the observations in Table 3.2. In the absence of novobiocin (0 μM), similar growth is observed at 24 hours for both wild type and Δ*acrAB E.coli* strains, transformed with the monatin operon (*aspC, proA*) on the pProNde vector. However in the presence of 40 or 80 ppm novobiocin, growth is completely inhibited for the Δ*acrAB E.coli* strain, while the corresponding control exhibited only slight inhibition of growth. In the presence of the AcrAB induced, sodium decanoate, the corresponding control strain grew to similar optical densities with 0 ppm, 40 ppm or 80 ppm

novobiocin, while growth of the Δ*acrAB E.coli* strain was completely inhibited.

EXAMPLE 4

Strategy to Identify Monatin Transporters from *E.coli, Corynebacterium* sp. or Other Microorganisms

[0091] Data from other examples in this application showed that the AcrAB and EmrAB multidrug efflux pumps are capable of transporting monatin. Induction of the AcrAB transport system with decanoic acid, resulted in a further increase in monatin efflux. In addition to the *acrAB* and *emrAB* transport system genes, numerous transporter genes can be identified using putative membrane topology inference and bioinformatics approaches. It has been reported that the *E.coli* genome encodes at least twenty drug transport systems that can confer drug resistance when overexpressed.

[0092] It is possible that some of these transporters might not be expressed from their native promoters or their expression might be repressed by general or specific repressor molecules in their native hosts. Nishino, K., and Yamaguchi, A., "Analysis of a complete library of putative drug transporter genes in *Escherichia coli*," *J. Bacteriol. 183*:5803-5812, (2001). It is also reported that some of these transporters with the exception of *acrAB* are not optimally expressed under normal fermentation/cultivation conditions (Sulavik, M.C., *et al.*, "Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes," *Antimicrob. Agents Chemother. 45*:1126-1136, (2001)), and thus special methods would need to be employed to detect the activity of these transporters. Based on the information above, additional transporters in *E.coli* as well as other microorganisms might be capable of transporting monatin with varying degrees of efficiency and selectivity.

[0093] Bioinformatics approaches (look for specific transporter characteristics, trans-membrane domains etc), public domain literature searches etc. can be used to identify sources of transporter gene candidates and also provide information about inducers for transporters. Transporters can be grouped into previously identified classes (acknowledged by experts). One or

two members from each class could be identified to determine their role in monatin transport. This strategy has the potential to permit extrapolation of observations from representatives tested, to the entire transporter class.

[0094] For example the monatin operon (*aspC, proA*) can be cloned into a vector and transformed into host microorganisms which are deficient in a specific transporter, or transporter system or individual transporter components. The specific transporter mutants with the capability to make monatin can be screened for loss of monatin transport compared to the appropriate wild-type controls. Transporter deletions that result in a decrease or loss of monatin transport indicate that the respective transporter might play a role in monatin efflux.

[0095] For example the monatin operon (*aspC, proA*) can be cloned into a vector and transformed into host microorganisms each of which is engineered to overexpress a specific transporter, or transporter system or individual transporter components. Microbial strains that show an increase in monatin transport compared to wild-type control with no overexpression of the transporter genes indicate that the respective transporter might play a role in monatin efflux.

[0096] Specific growth conditions or global inducers can increase the monatin efflux activity of transporter systems. Inducers can be transporter specific or general, and are beneficial because these inducers increase the activity of transporters making it easier to screen for activity with monatin transport. For example, indole increases the expression of a number of transporter genes including *acrD, acrE, cusB, emrK, mdtA, mdtE,* and *yceL.* Hirakawa, H. *et al.*, "Indole induces the expression of multidrug transporter genes in *Escherichia coli*," *Molecular Microbiology* 55:113-1126, (2005). Comparison of monatin efflux in induced systems with corresponding uninduced controls permits the evaluation of transporter systems and inducers capable of monatin transport. See, for example, Examples 1, 2, and 14.

[0097] For example the monatin operon (*aspC, proA*) can be cloned into a vector and transformed into appropriate host microorganisms. Monatin producing strains can be treated with appropriate inducers and checked for increase in monatin efflux. Microarray analysis can be used to identify the transporter genes that are overexpressed under induction conditions that result

in increased monatin efflux. These transporter gene candidates can be overexpressed to determine role in monatin efflux as described above.

[0098] For example the monatin operon (*aspC, proA*) can be cloned into a vector and transformed into host microorganisms deficient in one or more known transporters for monatin such as the AcrAB or the EmrAB systems. Induction of monatin transport in a host background that is lacking some of the major known transporters will permit the detection of additional monatin transporters in wild-type microorganism or in strains which are engineered to overexpress a specific transporter, or transporter system or individual transporter components. Microbial strains that show an increase in monatin transport compared to appropriate control strains under the same induction conditions indicate that the respective inducer/transporter might play a role in monatin efflux.

[0099] In addition to monatin efflux observed in the examples described in this application, we also observed red color formation in the culture medium, presumably due to reactions involving monatin intermediates such as indole-3-pyruvate ("I3P"), in the culture medium indicating that monatin intermediates are also being transported.

[0100] The indole-3-pyruvate efflux with resultant color formation (due to I3P complex formation) could be used as a screen for I3P transport. Given similarities in structure with monatin and monatin intermediates like indole-3-pyruvate, transport systems capable of I3P transport could be candidates for monatin transport (assumption that transporters may not discriminate between I3P and Monatin). For example, *Streptomyces griseoglavus* is an active producer of cellular and extra-cellular indole-3-pyruvate and would be a good candidate organism to screen for I3P and monatin transporters. El-Abyad, M.S., and Farid, M., "Optimization of culture conditions for indole-3-pyruvic acid production by *Streptomyces griseoflavus*," *Can. J. Microbio. 40*:754-760, (1994). Increasing the efficiency of monatin efflux would require modification of candidate transporters to increase specificity for monatin transport by reducing transport of monatin intermediates/precursors like indole 3-pyruvate and monatin as well as initial substrates like tryptophan or pyruvate.

EXAMPLE 5

Cloning and Overexpression of TolC

[0101] This example describes methods that were used to clone and overexpress *E. coli tolC* gene.

[0102] **Polymerase Chain Reaction Protocol:** Primers were designed with 5' restriction sites and overhangs for cloning into the pProNco vector (Clontech, Palo Alto, CA). primers: N term: 5'-GGCCTTGGCCATGGAAATGAAGAAATTGCTCCCC -3' and C term: 5'-CCGGCCAAGCTTTCAGTTACGGAAAGGGTTAT-3' . The *tolC* gene was amplified using the following PCR protocol. In a 50 µL reaction 0.150 µg template (*E. coli* MG1655), 1.6 µM of each primer, 0.4 mM each dNTP, 2.8 U Expand High Fidelity™ Polymerase (Roche, Indianapolis, IN), 0.5 U Pfu polymerase (Stratagene, La Jolla, CA), 1X Expand™ buffer with Mg, and 2.5 µL DMSO were used. The thermocycler program used included a hot start at 94 °C for 3 minutes, 8 repetitions of the following steps: 94 °C for 30 seconds, 52 °C for 45 seconds, and 72 °C for 2 minutes 30 seconds, followed by 18 repetitions of the following steps: 94 °C for 30 seconds, 59 °C for 45 seconds, and 72 °C for 2 minutes 30 seconds. After the 22 repetitions the sample was maintained at 72 °C for 7 minutes and then stored at 4 °C. This PCR protocol produced a product of 1475 bp.

[0103] **Cloning of *tolC* gene:** The PCR product was gel purified from 0.8% TAE-agarose gel using the Qiagen gel extraction kit (Valencia, CA). The PCR product was quantified using a SmartSpec 3000™ spectrophotometer. The product was TOPO Blunt cloned following manufacturer's recommended protocols (Invitrogen, Carlsbad, CA). Transformants were PCR screened to confirm TolC insert using protocol described above. Verified TOPO clones were digested with restriction enzymes *NcoI* and *HindIII* following the manufacturer's recommended protocols (New England Biolabs, Beverly, MA); the 1.475 kb band was gel purified from 0.8% TAE-agarose gel using the Qiagen gel extraction kit. Vector pProNco was prepared by digestion with restriction enzymes *NcoI* and *HindIII* followed by treatment with shrimp

alkaline phosphatase and purification from 0.8% TAE-agarose gel using the Qiagen gel extraction kit.

[0104] The digested vector and inserts were ligated using the Rapid ™ DNA Ligation Kit (Roche, Indianapolis, IN). Approximately 50 ng of treated insert, 100 ng of treated vector (3 to 1 molar ratio of insert to vector), 5 U of T4 DNA ligase, and 1X ligation buffer were incubated for 5 minutes at room temperature. The ligation reactions were cleaned up using the High Pure PCR Product Purification Kit (Roche) and used to transform *E. coli* DH10B electrocompetent cells (Invitrogen, Carlsbad, CA). 10 μL of each ligation reaction was added to 40 μL of DH10B cells, which were transformed by electroporation using the BioRad Gene Pulsar II under the following conditions: 2.5 kV, 25 μF, 200 ohm in a 0.2 cm cuvette. The cells were allowed to recover in 1 mL of room temperature SOC for 1 hour at 37 °C with shaking at 225 rpm. Cells were plated on LB plates containing kanamycin (50 μg/mL); plates were incubated at 37 °C overnight.

[0105] Plasmid DNA was purified from the resulting transformants using the Qiagen spin miniprep kit and screened for the correct inserts by restriction digest with *NcoI* and *HindIII*. The sequences of plasmids appearing to have the correct insert were verified by dideoxy chain termination DNA sequencing.

[0106] ***tolC* Gene Expression:** Plasmid DNA, verified by sequence analysis, was subcloned into *E. coli* expression host BL21(DE3) (Novagen, Madison, WI). The cultures were grown and the plasmids were isolated using Qiagen miniprep kit, and analyzed by restriction digest to confirm identity. Cultures were grown in 50 mL LB containing kanamycin (50 mg/L) at 30 °C , 225 rpm to an OD_{600} of 0.5 – 0.6 and induced with 100 mM IPTG (isopropyl thiogalacatoside) and 0.5% arabinose for overexpression of the *tolC* gene. The effect of overexpression of TolC on monatin transport is described in Examples 6 and 7 below.

EXAMPLE 6

Excretion of Monatin Increased with *tolC* Overexpression in *E.coli*

[0107] In Gram-negative bacteria, drug resistance is due in part to the activity of transmembrane efflux-pumps, which are composed of three types of proteins. A representative pump from *Escherichia coli* is an assembly of the trimeric outer-membrane protein TolC, which is an allosteric channel, the trimeric inner-membrane proton-antiporter AcrB, and the periplasmic protein, AcrA. The pump transports substrates outside from the bacterium using proton electrochemical force. Fernandez-Recio, J., *et al.*, "A model of a transmembrane drug-efflux pump from Gram-negative bacteria," *FEBS Lett. 578*:5-9, (2004).

[0108] The *tolC* gene in *E.coli* encodes an outer membrane protein that functions in conjunction with several different efflux pumps. TolC plays an active role in transport of various substrates from Gram-negative bacteria such as *E.coli* and *Pseudomonas aeruginosa*. TolC homologs are ubiquitous among Gram-negative bacteria and approximately a hundred TolC homologs have been identified. Dinh, T. *et al.*, *J. Bacteriol. 176*:3825-3831, (1994); Johnson, J. and Church, M. *J. Mol. Biol. 287*:695-715, (1999); Anderson, C. *et al.*, *EMBO Rep. 1*:313-318, (2000). The *tolC* gene was overexpressed in *E.coli* to determine if an increase in availability of the TolC channel would increase monatin transport.

[0109] *E.coli* strains BL21 (DE3) with the monatin operon (*aspC*, aspartate aminotransferase and *proA*, aldolase genes) and the pProNde plasmid (pProLAR from Clontech, modified as described in US20040235123) either with or without the *tolC* gene were tested for monatin transport.

[0110] Strains used for the experiment included *E.coli* BL21 (DE3):: *aspCproA*pET32 and *tolC* pProNde or pProNde without *tolC*. For inoculum, the *E.coli* strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 100 μg/mL ampicillin and 50 μg/mL kanamycin.

[0111] For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman, *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as

follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt, F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746, (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ $-6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0112] For treatments, 3-4 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 100 μg/mL ampicillin and 50 μg/mL kanamycin. Conditions for the treatments included 30 °C throughout the experiment and 250 rpm agitation. At 0.4 OD_{600nm} , induction of the plasmid genes was initiated. At induction, 0.5 mM IPTG, 0.5% arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mL of Balch's vitamins (Balch, W.E., *et al.*, "Methanogens: reevaluation of a unique biological group," *Microbiol. Rev. 43*:260-296, (1979)) were added. Additions of 1 g L-tryptophan, 5 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3 hours following induction. Some treatments included 2.5 mM sodium decanoate addition at 3 hours following induction. Samples for monatin and dry cell weight determination were taken at 6.5, 25 and 50 hours.

[0113] The amount of monatin excreted was determined using the method described in Example 13.

Table 6.1

Monatin per dry cell weight excreted by *E.coli*

	Monatin / dry cell weight (mg monatin /g dcw)	
Strain	**25 hours**	**50 hours**
E.coli BL21 DE3 *aspCproA*pET32 & pProNde w/o *tolC* (control)	nd	nd
E.coli BL21 DE3 *aspCproA*pET32 & pProNde w/o *tolC* (control)	0.11	0.09
E.coli BL21 DE3 *aspCproA*pET32 & *tolC*pProNde	0.69	0.50
E.coli BL21 DE3 *aspCproA*pET32 & *tolC*pProNde	1.18	0.86

nd: not detected

Table 6.2

Average monatin per dry cell weight excreted by E.coli

	Monatin / dry cell weight (mg monatin / g dcw)	
Strain	**25 hr average**	**50 hr average**
E.coli BL21 DE3 *aspCproA*pET32 & pProNde w/o *tolC* (control)	0.056	0.043
E.coli BL21 DE3 *aspCproA*pET32 & *tolC*pProNde	0.939	0.679

[0114] As described above, the strain with TolC overexpression excreted 0.939 mg monatin per g dry cell weight versus 0.056 mg/g without *tolC* overexpression at the 25 hour sampling point. This is a 16.8 fold increase in monatin transporter in a strain that has increase availability of the TolC channel. A similar trend was obtained at the 50 hour sampling point with a 15.8 fold increase in monatin transported. These data show that more monatin is transported in *E.coli* strains that have *tolC* gene overexpressed.

EXAMPLE 7

Excretion of Monatin Increased with *tolC* Overexpression in Combination with Induction of the AcrAB Efflux Pump in *E.coli*

[0115] It was demonstrated above that the AcrAB multidrug efflux pump could be induced in *E.coli* by the addition of 2.5 mM sodium decanoate. In

this example, the combination of induction of the AcrAB efflux pump with increased availability of the TolC channels was evaluated. *E.coli* strains overexpressing the *tolC* gene were additionally subjected to treatment with 2.5 mM sodium decanoate to simultaneously induce the AcrAB pumps.

[0116] The amount of monatin excreted was determined using the method described in Example 13.

Table 7.1

2.5 mM sodium decanoate treatment: Monatin per dry cell weight excreted by *E.coli*

	Monatin / dry cell weight (mg monatin / g dcw)	
Strain	25 hr	50 hr
E.coli BL21 DE3 *aspC proA*pET32 & pProNde w/o *tolC* (control)	0.17	0.11
E.coli BL21 DE3 *aspC proA*pET32 & pProNde w/o *tolC* (control)	0.19	0.09
E.coli BL21 DE3 *aspC proA*pET32 & *tolC*pProNde	8.88	7.99
E.coli BL21 DE3 *aspCproA*pET32 & *tolC*pProNde	12.19	10.23

Table 7.2

2.5 mM sodium decanoate treatment: Average monatin per dry cell weight excreted by *E.coli*

	Monatin / dry cell weight (mg monatin / g dcw)	
Strain	25 hr average	50 hr average
E.coli BL21 DE3 *aspCproA*pET32 & pProNde w/o *tolC* (control)	0.18	0.10
E.coli BL21 DE3 *aspCproA*pET32 & *tolC*pProNde	10.53	9.11

Average of n=2 treatments

[0117] As shown in the tables above, in *E.coli* strains under conditions that have the AcrAB transport system induced by sodium decanoate treatment, combined with the overexpression of the *tolC* gene, there was a 58.5 and 91.1 fold increase in monatin transport, over the treatment without *tolC* overexpression at the 25 and 50 hour sample points, respectively. These data demonstrated the additional advantages of combining overexpression of the

tolC gene with induction of the AcrAB transport system for increased monatin efflux.

EXAMPLE 8

Corynebacterium glutamicum Glutamate Auxotroph (Deficient) Strains have Increased Monatin Excretion and/or Production

[0118] *Corynebacterium glutamicum* ATCC strain 13032 is a glutamate-producing strain. The $NADP^+$ - dependent isocitrate dehydrogenase gene (ICD; EC 1.1.1.42, Gen bank accession number X71489) is one of the key enzymes of the citric acid cycle and converts D-isocitrate to 2-oxoglutarate, CO_2 and NADPH. 2-oxoglutarate can be further reductively aminated to form glutamate. Inactivation of the *icd* gene resulted in glutamate auxotrophy. Eikmanns, B.J., *et al.*, "Cloning, sequence analysis, and inactivation of the *Corynebacterium glutamicum icd* gene encoding isocitrate dehydrogenase and biochemical characterization of the enzyme," *J Bacteriol., 177*: 774-782, (1995). Two *icd* mutants were obtained from Prof. Hermann Sahm (Institut fur Biotechnologie des Forschungszentrums Julich, Germany.). Glutamate auxotrophy was confirmed by the inability of the *icd* mutants to grow in minimal media without glutamate supplementation. The *icd* mutants were transformed with the monatin operon (*aspC /proA*) located on the pEKEX-2 vector (Eikmanns, *et al.*, *Gene 102*: 93-98, (1991)) hereafter referred to as APpEKEX-2. Induction of the monatin operon resulted in monatin production and excretion outside the cell.

[0119] *C. glutamicum* 13032 strains (with or without the inactivated *icd* gene) transformed with APpEKEX-2 were grown overnight in LB medium supplemented with 5 μg/mL chloramphenicol at 30 °C and 250 rpm. For the experimental treatment flasks, 100 mL of Kraemer's A medium was used in each shake flask. Hoisted C., and Kraemer, R., "Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*," *Arch. Microbiol 151*:342-347, (1989). Kraemer's A medium contained (per liter): 5 g $(NH_4)_2SO_4$, 5 g urea, 2g KH_2PO_4 , 1.53 K_2HPO_4, 0.249 g $MgSO_4*7H_2O$, 50 g glucose, 0.01 g $FeSO_4$-$7H_2O$, 0.01 g $MnSO_4$-H_2O, 0.01 g $CaCl_2$-$2H_2O$, 0.03 mg $ZnSO_4$-$7H_2O$, 0.1mg H_3BO_3 0.07 mg $CaCl_2$-

$6H_2O$, 0.01 mg $NiCl_2$-$2H_2O$, 0.03 mg $CuCl_2$-$2H_2O$, 0.1 mg as Mo^{+6} from $(NH_4)_6Mo_7O_{24}$-$4H_2O$ and 1 μg biotin. The pH was adjusted to 7.0. All flasks (with the glutamate auxotroph strain (*icd2*) as well as the wild-type control) were supplemented with 5 mM glutamate.

[0120] For treatments, 4-7 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks. Conditions for the treatments included 30 °C throughout the experiment and 250 rpm agitation. At 0.4-0.7, OD_{600nm} induction of the monatin operon genes was initiated. 0.5 mM IPTG was used for induction and 0.5 mM pyridoxine hydrochloride, and 0.04 mM pyridoxal-5'-phosphate ("PLP") were added at the time of induction. Additions of 1 g L-tryptophan, 5 g/L sodium pyruvate and 10 μg/mL ampicillin were made 3 hours following induction. Samples for monatin and dry cell weight determination were taken at about 24, and 48 hours after inoculation (run time).

Table 8.1

Monatin per unit biomass for glutamate auxotrophs and control

		Run hour	
Strain	**Strain type**	**24.5**	**48**
C. glutamicum 13032 icd2 no76:: APpEKEX2	glutamate auxotroph	4.01	15.97
C. glutamicum 13032 control on APpEKEX2 (5 mM glutamate)	Wild-type control	0.16	0.28

Results for monatin / dry cell weight in mg monatin/g dcw
n=3 for both treatments at all sample times

[0121] Because monatin has a glutamate backbone, one possible candidate for the transport of monatin outside the cell could be a glutamate efflux transporter. However, to date a glutamate transporter has not been identified in Corynebacteria. In the event that a glutamate transporter could transport monatin, without being bound by theory, one might expect that without the competition for glutamate transport in the case of a glutamate auxotroph, more monatin could be effluxed by the transporter. In addition, pyruvate is an

intermediate for both glutamate and monatin production in *Corynebacterium glutamicum*. Because there is a high carbon flux to glutamate in the glutamate-producing bacterium, the use of *Corynebacterium* strains that are deficient in glutamate production could result in increased conversion of the pyruvate to monatin. ICD enzymes have roles in both energy production and intermediary metabolism (Eikmanns, B.J., *et al.*, "Cloning, sequence analysis, and inactivation of the *Corynebacterium glutamicum icd* gene encoding isocitrate dehydrogenase and biochemical characterization of the enzyme," *J. Bacteriol. 177*: 774-782, (1995)) and thus there could be additional advantages for monatin production in a strain with an inactivated *icd* gene. One additional possibility is that the accumulation of intermediates from reactions upstream of ICD might activate the formation of an efflux pump that prevents the build up of these intermediates and that this efflux pump is capable of monatin transport.

[0122] The *C. glutamicum* 13032 *icd*2 glutamate auxotroph transformed with the monatin operon (*aspCproA*pEKEX-2) produced an average of 15.97 mg monatin per gram of dry cell weight compared to 0.28 mg monatin per gram of dry cell weight for the wild type control with the monatin operon. These results showed that *C. glutamicum* 13032 glutamate auxotrophs with an inactive *icd* gene, transformed with the monatin operon excreted 57 fold more monatin than wild type strains with a functional *icd* gene.

EXAMPLE 9

E.coli Glutamate Auxotroph (*icdA* Deficient) Strains should have Increased Monatin Excretion Potential

[0123] While screening mutants of *E.coli* and other bacteria on nutrient plates with low levels of nalidixic acid, it was reported that resistance was found to result from mutations at different genetic loci. One such locus is the *icdA* gene encoding isocitrate dehydrogenase. A mutation in the *icdA* gene results in glutamate auxotrophy and accumulation of large amounts of citrate and isocitrate, the intermediates before the reaction catalyzed by IcdA. The association of intermediate accumulation and nalidixic acid resistance is predicted as follows: the metabolites/intermediates activate the formation of

an efflux pump that removes nalidixic acid from the cell and thus prevents toxicity. It was reported that there was increased *acrAB* transcription in the *icdA* mutant and demonstrated that in an *E.coli icdA* mutant, the expression of nalidixic acid resistance required the AcrAB-TolC efflux pump. Helling, R.B., *et al.*, "Toxic waste disposal in *Escherichia coli*," *J Bacteriol. 184*:3699-3703, (2002). Thus in an *E.coli* strain that had a mutation in *icdA* and transformed with the genes for monatin production, one would expect an increase in monatin transport due to an induction of the AcrAB-TolC transporter.

EXAMPLE 10

Malate as the Carbon Substrate Increased Monatin Excretion/Efflux in *E.coli*

[0124] Monatin has a glutamate backbone or can be considered to be a 4-substituted glutamate derivative. Monatin may therefore be transported out of a cell, for example, a bacterial cell, by transporters that translocate glutamate in exchange for substrates like malate. The glutamate/malate transporter in *Arabidopsis* plastids, encoded by the DiT2 gene, translocates glutamate and malate in antiport manner. Renne, P., *et al.*, "The Arabidopsis mutant dct is deficient in the plastidic glutamate/malate translocator DiT2," *Plant J. 35*:316-331, (2003); Taniguchi, M., *et al.*, "Identifying and Characterizing Plastidic 2-Oxoglutarate/Malate and Dicarboxylate Transporters in *Arabidopsis thaliana*," *Plant and Cell Physiology*, 2002, *43*:706-717, (2002). The glutamate/malate transporter family is homologous with the 2-oxoglutarate / malate transporter in spinach chloroplasts which is related to the CitT transporter in *E.coli* that is believed to be an antiporter for citrate and succinate. Pos, K.M., "The *Escherichia coli* citrate carrier CitT: a member of a novel eubacterial transporter family related to the 2-oxoglutarate/malate translocator from spinach chloroplasts," *J. Bacteriol. 180*:4160-4165, (1998). It is reported that the *E. coli* CitT protein is a member of a novel family of eubacterial transporters involved in the transport of di- and tricarboxylic acids. Monatin is a dicarboxylic acid. There is a possibility that this transporter could permit a host to take up malate while excreting glutamate or monatin into the

supernatant. Another possibility for malate functioning to increase monatin transport could be due to the role that malate might play as an alternative carbon source affecting growth rate and carbon distribution to metabolic pathways differently than with glucose. Malate can also be converted to pyruvate internally by malic enzymes encoded by 2 genes (Fischer, E., and Sauer, U., "Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS," *Eur J Biochem. 270*:880-891, (2003)), and, because pyruvate is one of the precursors to monatin, a greater availability of pyruvate may result in increased monatin production and consequently increased monatin secretion. Growth of *E. coli* on malate as the primary carbon source resulted in increases in monatin excretion into the culture medium.

[0125] For inoculum, *E.coli* BL21 DE3 *aspC / proA* on pET30 was grown overnight in LB medium with 50 μg/mL kanamycin at 37 °C and 250 rpm. For the experimental treatments, Trp-1 medium, as described in Example 6 (Zeman, *et al. Folia Microbiol. 35*:200-204, (1990)), and 50 μg/mL kanamycin was used. Malate and glucose were 4 g/L initially for their respective treatments. For treatment flasks, 2.2 v/v % of inoculum was added to 50 mL medium volume in 250 mL baffled shake flasks. For glucose treatments, induction of the monatin operon was initiated at 0.8 OD_{600nm}. For malate treatments, induction was at 0.25 OD. At induction, 0.5 mM IPTG, 0.5 mM pyridoxine hydrochloride, and 0.2 mL of Balch's vitamins (Balch, W.E., *et al.*, "Methanogens: reevaluation of a unique biological group," *Microbiol. Rev. 43*:260-296 (1979)) were added. Temperature was reduced to 30 °C at induction. Additions of 1 g L-tryptophan, 5 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP"), 10 μg/mL ampicillin, and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3 hours following induction. Samples for monatin and dry cell weight determination were taken at 24 hours post induction.

Table 10.1

Summary table for *E.coli* with glucose or malate as carbon source

		Monatin / dcw (mg /g)	
Treatment no.	Carbon source	24 hours	24 hour average
1	glucose	6.39	
2	glucose	6.17	6.28
3	malate	7.19	
4	malate	8.25	7.72

[0126] From the results shown above, the monatin transported per unit biomass was increased with the malate treatment when compared to the glucose treatment (7.72 to 6.28). Malate as the primary carbon source resulted in 23% higher monatin transported per unit biomass. Using malate as either a primary or supplementary carbon source in combination with the other conditions described that have been shown or expected to increase monatin efflux, could result in further increases in monatin production and excretion.

EXAMPLE 11

Increased Monatin Excretion by Putative Efflux Transporter ("PET") Aae Transporter Deletion in *E.coli*

[0127] A family of putative efflux transporters ("PET") has been reported in bacteria, yeast and plants. The PET family members with accession numbers, identified in *E.coli* include, YjcQ (P32715), YccS (P75870), YhfK (P45537) and YhcP (P46481). Only one of the members of the PET family, AaeAB/YhcP, has been functionally characterized to date. Van Dyk, T.K., *et al.*, "Characterization of the *Escherichia coli* AaeAB efflux pump: a metabolic relief valve?," *J Bacteriol.* 2004 Nov; *186*:7196-7204, (2004) The bacterial and yeast proteins display a duplicated internal repeat element consisting of an N-terminal hydrophobic sequence of about 170 residues, exhibiting six putative alpha-helical transmembrane spanners (TMSs), followed by a large, C-terminal, hydrophilic, cytoplasmic domain. The plant proteins exhibit only one such unit, but they have a larger C-terminal cytoplasmic domain.

Arabidopsis thaliana encodes at least seven paralogs of the PET family. The Gram-negative bacterial proteins are sometimes encoded by genes found in operons that also contain genes that encode membrane fusion proteins. This fact strongly suggests that PET family proteins are efflux pumps. Harley, K.T., and Saier, M.H. Jr., "A novel ubiquitous family of putative efflux transporters," *J Mol Microbiol Biotechnol. 2*:195-198, (2000).

[0128] The PET mutant strains used for the experiments were obtained from Prof. Milton Saier at the University of California at San Diego and included *E.coli* BW 25113 wild type and the four single knockout mutants *E.coli* BW 25113 Δ*yhcP*, *E.coli* BW 25113 Δ*yccS*, *E.coli* BW 25113 Δ*yjcQ*, *E.coli* BW 25113 Δ*yhfK* and the quad mutant *E.coli* BW 25113 Δ*yhcP* Δ*yccS* Δ*yjcQ* Δ *yhfK*. All strains were transformed with the monatin operon genes *aspC* and *proA* on the pProNde vector as described in other examples in this application. The strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 50 μg/mL kanamycin.

[0129] For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman, *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution (Neidhardt, F.C., *et al.* "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746, (1974)). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ $-6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0130] For monatin production shake flasks, 3-4 % v/v of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 50 μg/mL

kanamycin. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then, 30 °C following induction. At 0.6 OD_{600nm}, induction of the plasmid genes was initiated. At induction, 0.5 mM IPTG, 0.5% arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mL of Balch's vitamins were added. Additions of 1 g L-tryptophan, 5 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP"), 10 μg/mL ampicillin and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3.5 hours following induction. Samples for monatin and dry cell weight determination were taken at 24 and 30 or 31 hours. Monatin was measured by LC/MS/MS as described in Example 13. The results are shown below.

Table 11.1

Average monatin per dry cell weight for 30 hour samples

Strain	30 hour average monatin / dcw (mg/g)
E coli BW 25113 wild type:: *aspCproA*pProNde	1.81
E.coli BW 25113 Δ*yhcP*:: *aspCproA*pProNde	2.02
E.coli BW 25113 Δ*yccS*:: *aspCproA*pProNde	1.86
E.coli BW 25113 Δ*yjcQ*:: *aspCproA*pProNde	1.92
E.coli BW 25113 Δ*yhfK*:: *aspCproA*pProNde	1.60
E.coli BW 25113 Δ*yhcP* Δ*yccS* Δ*yjcQ* Δ*yhfK* :: *aspCproA*pProNde	6.16

n=2 for all 6 strains

[0131] The quad PET mutant strain *E.coli* BW 25113 Δ*yhcP*(*aaeB*) Δ*yccS* Δ*yjcQ* Δ*yhfK* excreted significantly more monatin per dry cell weight, 6.16, than either the wild type or four single mutants which all averaged in the range of 1.6 – 2.02 mg monatin /g dry cell weight.

[0132] The YhcP efflux system has been reported to have a high degree of specificity to certain hydroxylated, aromatic carboxylic acids. The narrow specificity of the AaeAB (YhcP) efflux system is in distinct contrast to multidrug efflux systems such as AcrAB-TolC. Van Dyk, T.K., *et al.*,

"Characterization of the *Escherichia coli* AaeAB efflux pump: a metabolic relief valve?," *J. Bacteriol. 186*:7196-7204, (2004). It has been suggested that the role of the AaeAB efflux system is as a "metabolic relief valve" and it is expected that if a metabolic upset/internal stress were to occur (such as an accumulation of monatin or monatin intermediates) the expression of the efflux system would be activated.

[0133] The fact that an increase in monatin efflux is observed in a quad PET (Aae) transporter deletion background, indicates a putative role of one or more of the PET transporters in efflux of one or more monatin intermediates. Thus in the strain that is engineered to produce monatin but has a combined inactivation of these four PET/Aae transporters, there could be a reduction in loss of monatin intermediates and consequently more monatin being produced by the cell and transported by transporter systems such as AcrAB TolC/ EmrAB TolC etc. Another possibility is that in a quad PET mutant background, there is greater accumulation of a metabolite that may or may not be related to monatin biosynthesis that then induces a monatin transporter, resulting in increased monatin efflux.

[0134] Various deletion combinations of the individual PET transporters could be as effective or more effective for monatin efflux than the quad PET transporter deletion strain. One could combine the quad PET mutant background with any of the other transporters or conditions shown to increase monatin transport to generate a strain with additional potential for monatin transport.

[0135] The results above demonstrated that either through a direct or indirect mechanism, an *E.coli* strain with combined deletions of the YhcP (AaeB), YccS, YjcQ and YhfK putative efflux transporters (PETs), excreted more monatin per dry cell weight than the corresponding wild-type control strain.

EXAMPLE 12

Transformation of *Corynebacterium glutamicum*

[0136] Where the specification refers to transformation of *Corynebacterium glutamicum*, the following method was used.

[0137] Electrocompetent cells of *C.glutamicum* were prepared by inoculating starter culture (grown overnight) into 200 ml MB medium (5 g/L yeast extract, 15 g/L tryptone, 5 g/L soytone, 5 g/L sodium chloride) to an initial OD_{600nm} of 0.1. Cultures were incubated at 200 rpm to OD_{600nm} of 0.7 and cells were collected by centrifugation at 4 °C. The cell pellet was washed 3 times with 40 ml ice-cold buffer (20 mM HEPES, pH 7.2, containing 5% glycerol). The cell pellet is then washed 2 times with 20 ml ice-cold 10% v/v glycerol and the pellet is resuspended in 1 ml 10% v/v glycerol. The washed electrocompetent cells are divided into 150 µL aliquots and stored frozen at –80 °C.

[0138] Before transformation of the electrocompetent *C. glutamicum* cells, 150 µL electrocompetent cells were thawed on ice. 1 µg of the desired plasmid, was added to the cells and incubated on ice for 5 minutes, and then transferred to a chilled 0.2 cm cuvette. The cells were overlaid with 0.8 mL ice-cold 10% glycerol on top of cell suspension, being careful to avoid mixing of layers and electroporated at 200 ohms, 25 uFd, 12.5 kV/cm. The cell suspension was transferred to 4 mL of pre-warmed 46 °C MB medium and incubated at 46 °C for 6 minutes without shaking. Cell suspensions were incubated at 30 °C, 200 rpm for 50 min before plating on MB plates containing appropriate selective antibiotic and incubation at 30 °C to allow the growth of transformed *C. glutamicum* strains.

EXAMPLE 13

Method of Detecting Monatin and Monatin Stereoisomers

[0139] This example describes methods used to detect the presence of monatin, tryptophan and glutamic acid. It also describes a method for the separation and detection of the four stereoisomers of monatin.

LC/MS/MS Multiple reaction Monitoring ("MRM") Analysis of Monatin and Tryptophan

[0140] Analyses of mixtures for monatin and tryptophan derived from *in vitro* or *in vivo* biochemical reactions were performed using a Waters/Micromass liquid chromatography-tandem mass spectrometry ("LC/MS/MS") instrument

including a Waters 2795 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor placed in series between the chromatograph and a Micromass Quattro Ultima triple quadrupole mass spectrometer. LC separations were made using an Xterra MS C_8 reversed-phase chromatography column, 2.1mm x 250 mm at 40 °C. The LC mobile phase consisted of A) water containing 0.05% (v/v) trifluoroacetic acid and B) methanol containing 0.05% (v/v) trifluoroacetic acid.

[0141] The gradient elution was linear from 5% B to 35% B, 0-4 min, linear from 35% B to 60% B, 4-6.5 min, linear from 60% B to 90% B, 6.5-7 min, isocratic at 90% B 7-11 min, linear from 90% B to 95% B, 11-12 min, linear from 95% B to 5% B, 12-13 min, with a 5 min re-equilibration period between runs. The flow rate was 0.25 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of protonated molecular ions ($[M + H]^+$) of the analytes of interest, and production of characteristic fragment ions. The following instrumental parameters were used for LC/MS/MS Multiple Reaction Monitoring ("MRM") analysis of monatin and tryptophan: Capillary: 3.5 kV; Cone: 40 V; Hex 1: 20 V; Aperture: 0 V; Hex 2: 0 V; Source temperature: 100 °C; Desolvation temperature: 350 °C; Desolvation gas: 500 L/h; Cone gas: 50 L/h; Low mass resolution (Q1): 12.0; High mass resolution (Q1): 12.0; Ion energy: 0.2; Entrance: -5 V; Collision Energy: 8; Exit: 1V; Low mass resolution (Q2): 15; High mass resolution (Q2): 15; Ion energy (Q2): 3.5; Multiplier: 650. Five monatin-specific parent-to daughter MRM transitions are used to specifically detect monatin in *in vitro* and *in vivo* reactions. The transitions monitored are 293.1 to 158.3, 293.1 to 168.2, 293.1 to 211.2, 293.1 to 230.2, and 293.1 to 257.2. Tryptophan is monitored with the MRM transition 204.7 to 146.4. For Internal standard quantification of monatin and tryptophan, four calibration standards containing four different ratios of each analyte to d5-tryptophan and d5-monatin, are analyzed. These data are subjected to a linear least squares analysis to form a calibration curve for monatin and tryptophan. To each sample is added a fixed amount of d5-tryptophan and d5-monatin, and the response ratios (monatin/d5-monatin;

tryptophan/d5-tryptophan) used in conjunction with the calibration curves described above to calculate the amount of each analyte in the mixtures.

Chiral LC/MS/MS (MRM) Measurement of Monatin

[0142] Determination of the stereoisomer distribution of monatin in *in vitro* and *in vivo* reactions was accomplished by derivitization with 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide ("FDAA"), followed by reversed-phase LC/MS/MS MRM measurement.

Derivitization of Monatin with FDAA

[0143] To 50 μL of sample or standard was added 200 μL of a 1% solution of FDAA in acetone. Forty μL of 1.0 M Sodium bicarbonate was added, and the mixture incubated for 1 h at 40 °C with occasional mixing. The sample was removed and cooled, and neutralized with 20 μL of 2.0 M HCl (more HCl may be required to effect neutralization of a buffered biological mixture). After degassing is complete, samples were ready for analysis by LC/MS/MS.

LC/MS/MS Multiple Reaction Monitoring for the Determination of the Stereoisomer Distribution of Monatin in in vitro and in vivo Reactions.

[0144] Analyses were performed using the LC/MS/MS instrumentation described in previous sections. LC separations capable of separating all four stereoisomers of monatin (specifically FDAA-monatin) were performed on a Phenomenex Luna 2.0 x 250 mm (3 μm) C18 reversed phase chromatography column at 40 °C. The LC mobile phase consisted of A) water containing 0.05% (mass/volume) ammonium acetate and B) acetonitrile. The elution was isocratic at 13% B, 0-2 min, linear from 13% B to 30% B, 2-15 min, linear from 30% B to 80% B, 15-16 min, isocratic at 80% B 16-21 min, and linear from 80% B to 13% B, 21-22 min, with a 8 min re-equilibration period between runs. The flow rate was 0.23 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of protonated molecular ions ($[M-H]^-$) of FDAA-monatin, and production of characteristic fragment ions.

[0145] The following instrumental parameters were used for LC/MS analysis of monatin in the negative ion ESI/MS mode: Capillary: 2.0 kV; Cone: 25 V;

Hex 1: 10 V; Aperture: 0 V; Hex 2: 0 V; Source temperature: 100°C; Desolvation temperature: 350°C; Desolvation gas: 500 L/h; Cone gas: 50 L/h; Low mass resolution (Q1): 12.0; High mass resolution (Q1): 12.0; Ion energy: 0.2; Entrance: -5V; Collision Energy: 20; Exit: 1V; Low mass resolution (Q2): 12; High mass resolution (Q2): 12; Ion energy (Q2): 3.0; Multiplier: 650. Three FDAA-monatin-specific parent-to daughter transitions are used to specifically detect FDAA-monatin in *in vitro* and *in vivo* reactions. The transitions are 543.6 to 268.2, 543.6 to 499.2, and 543.6 to 525.2. Identification of FDAA-monatin stereoisomers is based on chromatographic retention time as compared to purified monatin stereoisomers, and mass spectral data.

EXAMPLE 14

Induction with Salicylate Increased Monatin Transport

[0146] *C. glutamicum* ATCC 13032 strains transformed with *aspCProA*pEKEX-2 were grown overnight in LB medium supplemented with 25 μg/mL kanamycin incubated at 37 °C and shaking at 250 rpm. For the experimental treatment flasks, 100 mL of Kraemer's A medium was used in each shake flask. Hoisted C,, and Kraemer, R. "Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*," *Arch. Microbiol 151*:342-347, (1989). Kraemer's A medium contained (per liter): 5 g $(NH_4)_2SO_4$, 5 g urea, 2g KH_2PO_4 , 1.53 K_2HPO_4, 0.249 g $MgSO_4*7H_2O$, 50 g glucose, 0.01 g $FeSO_4$-$7H_2O$, 0.01 g $MnSO_4$-H_2O, 0.01 g $CaCl_2$-$2H_2O$, 0.03 mg $ZnSO_4$-$7H_2O$, 0.1 mg H_3BO_3 0.07 mg $CaCl_2$-$6H_2O$, 0.01 mg $NiCl_2$-$2H_2O$, 0.03 mg $CuCl_2$-$2H_2O$, 0.1 mg as Mo^{+6} from $(NH_4)_6Mo_7O_{24}$-$4H_2O$ and 1 μg biotin. The pH was adjusted to 7.0.

[0147] For treatments, 4.2 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks. Conditions for the treatments included 37 °C throughout the experiment and 250 rpm agitation. Sodium salicylate (0, 1 mM or 2 mM) was added 1 hour following inoculation. At 0.45-0.6 OD_{600nm} induction of the monatin operon genes was initiated. IPTG at 0.5 mM was used for induction, and additions at the time of induction

included 0.5 mM pyridoxine hydrochloride and 0.04 mM pyridoxal-5'-phosphate ("PLP"). Additions of 1 g L-tryptophan, 5 g/L sodium pyruvate and 10 μg/mL ampicillin were made 3 hours following induction. Samples for monatin and dry cell weight determination were taken at 23.5 and 48 hours.

Table 14.1

Salicylate induction increases monatin per dry cell weight

	Monatin / dcw (mg / g)	
sodium salicylate (mM)	23.5 hours	48 hours
0	4.34	9.30
1	7.41	9.94
2	7.54	9.82

[0148] MarA activates expression of the *mar* regulon, including *acrAB, tolC*, and *marRAB*, whereas MarR acts to downregulate this response by repressing the synthesis of MarA. The addition of some antibiotics, weak aromatic acids, such as salicylate, and a structurally diverse range of other compounds, such as the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone ("CCCP") have all been shown to cause induction of *mar* regulon and thus AcrAB and TolC expression. Grkovic, S., *et al.*, "Regulation of Bacterial Drug Export Systems," *Microbiology and Molecular Biology Reviews 66*:671-701, (2002).

[0149] In addition, the EmrAB multidrug pump of *E. coli* is induced in the presence of CCCP, the weak acid salicylate, and a number of other structurally unrelated drugs. The derepression is controlled by the EmrR, a MarR type of repressor protein. Cohen, S.P., *et al.*, "Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the *mar* operon and a *mar*-independent pathway," *J. Bacteriol. 175*:7856-7862, (1993); Lomovskaya, O., *et al.*, "EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB," *J. Bacteriol. 177*:2328-2334, (1995). Thus salicylate addition increases AcrAB and EmrAB transporter system activity in *E. coli*. Salicylate addition to *Corynebacterium* could induce homologs of AcrAB/EmrAB or other transporters resulting in increased monatin transport.

[0150] Thus treatment of *Corynebacterium glutamicum* with 1mM or 2mM sodium salicylate, resulted in an increase in the monatin transported. Monatin analysis was conducted as described in Example 13. A seven percent increase in monatin efflux was observed at 48 hours and a seventy percent increase in monatin transported at 23.5 hours.

EXAMPLE 15

Demonstration of Monatin Production and Excretion in *Pantoea stewartii*

[0151] Electrocompetent *Pantoea stewartii* (ATCC 8200) were prepared by culturing a 1% inoculum of *P. stewartii* cells in Nutrient broth from an overnight culture. Cells were incubated at 26 °C and 250 rpm to an OD 600 of ~0.6. The bacteria were pelleted by centrifugation (10 minutes at 10,000 x g) and washed in 50 ml of 10 mM HEPES (pH 7.0). The wash was repeated with 25 ml of 10 mM HEPES buffer (pH 7.0) followed by the same centrifugation protocol as above. The cells then were washed once in 25 ml of 10% glycerol. Following centrifugation, the cells were resuspended in 500 µL of 10% glycerol. Forty µL aliquots were frozen and kept at –80 °C until use.

[0152] pPROLarA.122 (ClonTech Laboratories, Inc.) was altered by site specific mutagenesis to introduce a *Nde* I restriction site at bp 132 (nucleotide numbering as described by ClonTech laboratories) and generated the vector pPRONde, by following the protocols as described in the Stratagene QuikChange site specific mutagenesis kit (Stratagene, Inc.) and using the mutagenic oligonucleotides (*Nde* I sites are underlined):

5'-GAGGAGAAAGGTACATATGGGTGAACAGAAAC-3'

5'-CAGTTTCTGTTCACCCATATGTACCTTTCTCC-3'

Thermocycler protocol:

1) 96 °C for 5 minutes
2) 96 °C for 30 seconds
3) 55 °C for 45 seconds
4) 72 °C for 3 minutes
5) Repeat steps 2-4; 24 times
6) 72 °C for 10 minutes

Recipe:	
10x Expand Polymerase Buffer	5 μL
dNTP's (10 mM each)	1 μL
pPRONde (~50 ng/uL)	0.1 μL
PCR primers (each)	0.5 μL
Expand Polymerase	0.5 μL
Water	42.4 μL
Total	50 μL

[0153] The resulting PCR product was purified by PCR clean-up kit (Qiagen) and digested with *Nde* I restriction endonuclease. The digested DNA was then purified by gel purification on a 0.8% agarose gel and ligated together. Ligation mixtures were precipitated by ethanol precipitation and digested with *Kpn*I restriction endonuclease in order to linearize any parental plasmid. The reaction mix was transformed into DH10B electrocompetent *E. coli.* Transformants were screened for removal of the *Kpn*I site by digestion with *Kpn*I restriction endonuclease.

[0154] A 29 bp section of the pPRONde vector was deleted using Quikchange site-directed mutagenesis (Strategene, La Jolla, CA), as the repetitiveness of this fragment within the vector had previously been shown to cause vector instability under stressful conditions. Primers for the mutagenesis were:

5'-ACGTCTGTGTGGAATTCTCGGACACCGAGGAG-3'

and

5'- CTCCTCGGTGTCCGAGAATTCCACACAGACGT-3'.

[0155] The mutagenesis was conducted as per manufacturer's protocol. Clones were screened by restriction digest with *Eco*RI, as a new *Eco*RI restriction site was created by deletion of the desired DNA fragment, and mutants were confirmed by sequencing. The resulting vector was named pPRONdeDel. The *aspC* gene was introduced into vector pPRONdeDel using restriction sites *Nde*I and *Bam*HI. The *proA* gene was subsequently introduced into vector pPRONdeDel using restriction sites *Bam*HI and *Not*I, resulting in vector *aspC* / *proA* / pPRONdeDel (APpPRONdeDel).

[0156] Vector APpPRONdeDel was transformed into electrocompetent *Pantoea stewartii* using a 0.2 cm cuvette and a Bio-Rad Gene Pulser II system as described in the Bio-Rad electroporation manual. The cells were allowed to recover in 900 μL SOC medium for 1 hour at 26 °C at 250 rpm. Cells were plated on LB-agar plates containing kanamycin (25 μg/mL).

[0157] For inoculum, the *P. stewartii* was grown overnight at 30 °C and 250 rpm in Luria-Bertani ("LB") medium with 25 μg/mL kanamycin. For the experimental treatments, Trp-1 + glucose medium, (Zeman, *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746, (1974)). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ $-6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2 - 4H_2O$, and 0.14 g $ZnSO_4 - 7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0158] For treatments, 3.5-5.0 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 25 μg/mL kanamycin. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then, 30 °C following induction. At 0.35-0.50 OD_{600nm}, induction of the plasmid genes was initiated. At induction, 1.0 mM IPTG, 0.5% L-arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mLs of Balch's vitamins were added. Additions of 10 g/L L-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3.0 hours following induction. Some treatments included 2.5 mM sodium decanoate and / or 10

μg/ml ampicillin addition at 3.0 hours following induction. Samples for monatin and dry cell weight determination were taken at 24, 30 and 48 hours.

Table 15.1

Monatin per dry cell weight excreted by *P. stewartii*

			Monatin / dcw (mg/g)		
Strain	Sodium decanoate (mM)	Tween 20 / ampicillin	24 hours	30 hours	48 hours
Pantoea stewartii :: *aspC / proA* / pProNde del	0	no Tween 20 / ampicillin	3.2	7.6	7.8
Pantoea stewartii :: *aspC / proA* / pProNde del	0	+ Tween 20 / ampicillin	4.9	8.4	37.1

[0159] Overexpression of the monatin operon, aspC-aminotransferase and proA- aldolase, in *Pantoea stewartii* results in increased excretion of monatin (monatin per dry cell weight). There is a 4 to 5-fold increase by 48 hours in monatin excretion when Tween 20 and ampicillin are added to the medium. Tween and ampicillin have been reported to stress the cell by affecting the cell envelope and thus assist with transport of metabolites outside the cell.

[0160] This is the first evidence of monatin production and export in the genus *Pantoea* and species *Pantoea stewartii.*

EXAMPLE 16

Increase of Monatin Excretion with Overexpression of the RobA Proteins

[0161] RobA is a member of the XylS/AraC subfamily of DNA binding proteins, and when overexpressed, has been shown to induce multiple antibiotic resistance in *Escherichia coli.* It has been reported that the multiple antibiotic resistance induced by the overexpression of RobA largely depends on the activation of the AcrAB efflux, as well as the activation of *micF.* Tanaka T., *et al.*, "RobA-induced multiple antibiotic resistance largely depends on the activation of the AcrAB efflux," *Microbiol Immunol. 41*:697-702, (1997). The MicF small RNA is encoded divergently from the gene encoding the OmpC porin and represses the expression of OmpF, another

porin. The exact role that MicF might play in monatin efflux remains to be determined.

[0162] The strains used for the experiment included *E. coli* MG1655:: *aspC* / *proA* / pProNdeDel together with the *robA* gene from *E. coli* cloned into the pUC19 vector. The control strain was *E. coli* MG1655:: *aspC* / *proA* / pProNde along with the pUC19 vector. The *robA* gene was amplified from *E.coli* W3110 using primers 5'TTAAGGCCGTCGACATGGATCAGGCCGGCATTAT3' and 5'TTCCAAGGTTGGATCCCTAAACGATGCGGCAGGC3', which introduced *SalI* and *BamHI* sites at the end of the amplified fragment. The PCR fragment was cloned between the *SalI* and *BamHI* sites of the vector pUC19 (GenBank/EMBL accession number L09137). For inoculum, the *E. coli* strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 100 μg/mL ampicillin and 50 μg/mL kanamycin.

[0163] For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746, (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ $-6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0164] For treatments, 3.5-5.0 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 100 μg/mL ampicillin and 50 μg/mL kanamycin. Conditions for the treatments included 250 rpm

agitation throughout and 37 °C up to induction, then, 30 °C following induction. At 0.30-0.50 OD_{600nm}, induction of the plasmid genes was initiated. At induction, 1.0 mM IPTG, 0.5% L-arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mLs of Balch's vitamins were added. Additions of 10 g/L L-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3 hours following induction. Some treatments included 2.5 mM sodium decanoate addition at 3 hours following induction. Samples for monatin and dry cell weight determination were taken at 24, 30 and 48 hours.

Table 16.1

Monatin per dry cell weight excreted by *E. coli*

		Monatin / dcw (mg/g)	
	Sodium decanoate (mM)	30 hours	48 hours
E.coli MG1655 :: *aspC proA* pProNdedel, pUC19 (control)	0	2.59	2.94
E.coli MG1655 :: *aspC proA* pProNdedel, *robA* pUC19	0	3.11	10.41

n=2 for all treatments

[0165] Overexpression of RobA without sodium decanoate treatment resulted in greater monatin excretion at 48 hours. Monatin per dry cell weight was 10.41 mg/g on average at 48 hours when RobA was overexpressed compared to 2.94 mg/g average with no RobA overexpression. RobA overexpression resulted in a 3.5-fold increase in monatin excretion. This is evidence that RobA overexpression had a positive impact on AcrAB expression or *micF* resulting in increased monatin excretion. The exact role that MicF might play in monatin efflux remains to be determined.

Table 16.2

Monatin per dry cell weight excreted by *E. coli*

		Monatin / dcw (mg/g)	
	Sodium decanoate (mM)	30 hours	48 hours
E.coli MG1655 :: *aspC proA* pProNdedel pUC19 (control)	2.5	4.66	4.31
E.coli MG1655 :: aspC proA pProNdedel, *robA* pUC19	2.5	6.91	25.62

n=2 for all treatments

[0166] Overexpression of RobA with 2.5 mM sodium decanoate treatment resulted in greater monatin excretion at 48 hours. Monatin per dry cell weight was 25.62 mg/g on average at 48 hours when RobA was overexpressed compared to 4.31 mg/g average with no RobA overexpression. RobA overexpression in the presence of sodium decanoate resulted in a greater than 5-fold increase in monatin excretion. This is evidence that RobA overexpression and decanoate addition had a positive and possibly synergistic impact on AcrAB expression or *micF* resulting in increased monatin excretion. The exact role that MicF might play in monatin efflux remains to be determined.

Table 16.3

Monatin per dry cell weight excreted by *E. coli* Δ*acrAB*

	Monatin / dcw (mg/g)		
	24 hours	30 hours	48 hours
E.coli MG1655 deltaAcrAB :: *aspC proA* pProNdedel, pUC19 (control)	10.7	29.7	39.3
E.coli MG1655 deltaAcrAB :: aspC proA pProNde del robA pUC19	45.7	58.6	81.7

n=2 for all treatments
2.5 mM sodium decanoate added to all treatments

[0167] Overexpression of RobA with 2.5 mM sodium decanoate addition in the *E.coli* MG1655 ΔAcrAB strain resulted in greater monatin excretion at 24, 30 and 48 hours. Monatin per dry cell weight was 81.7 mg/g on average at 48 hours when RobA was overexpressed in the ΔAcrAB strain compared to 39.3 mg/g average with no RobA overexpression in this strain. RobA overexpression in the ΔAcrAB strain with the presence of sodium decanoate resulted in a greater than 2-fold increase in monatin excretion. This is evidence that RobA overexpression and decanoate addition in the ΔAcrAB strain resulted in increased monatin excretion possibly due to action on the *micF* gene or transporter system other than the AcrAB transport system.

[0168] Taken together the data above conclusively demonstrated that RobA has a positive impact on monatin efflux, which could be through the activation of the AcrAB or other transport systems.

EXAMPLE 17

Increase of Monatin Excretion with Overexpression of the RamA Proteins

[0169] RamA, is a 113-amino-acid regulatory protein belonging to the AraC-XylS transcriptional activator family, in *Enterobacter aerogenes*. Overexpression of RamA was reported to induce an MDR phenotype in drug-susceptible *Escherichia coli* JM109 and *E. aerogenes* ATCC 13048, and resulted in an increased production of AcrA, a component of the AcrAB-TolC drug efflux pump. It was shown that RamA not only enhanced the transcription of the *marRAB* operon but was also able to induce a multi-drug resistance ("MDR") phenotype in a *mar*-deleted strain. Thus RamA is a transcriptional activator of the Mar regulon in addition to being a self-governing activator of the MDR cascade. Chollet R., *et al.*, "RamA is an alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*," *Antimicrob Agents Chemother.* *48*:2518-2523, (2004).

[0170] The strains used for the experiment included *E. coli* MG1655:: *aspC* / *proA* / pProNdeDel together with the *ramA* gene from *Enterobacter aerogenes* cloned into the pUC19 vector (GenBank/EMBL accession number L09137).

The control strain was *E. coli* MG1655:: *aspC* / *proA* / pProNde along with the pUC19 vector. The *ramA* gene was amplified from *E. aerogenes* ATCC 13048 by using primers, 5'GGCCGGTTAAGTCGACATGAATATATCCGCTCAGG3' and 5'TTAACCTTGGATCCTCAGTGCGCGCGGCTGT3', which introduced *Sal*I and *Bam*HI sites at the end of the amplified fragment. The PCR fragment was cloned between the *Sal*I and *Bam*HI sites of the vector pUC19 (GenBank/EMBL accession number L09137). For inoculum, the *E. coli* strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 100 μg/mL ampicillin and 50 μg/mL kanamycin.

[0171] For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746, (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ $-6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0172] For treatments, 3.5-5.0 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 100 μg/mL ampicillin and 50 μg/mL kanamycin. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then, 30 °C following induction. At an OD_{600nm} between 0.30-0.50, induction of the plasmid genes was initiated. At induction, 1.0 mM IPTG, 0.5% L-arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mLs of Balch's vitamins were added.

Additions of 10 g/L L-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3 hours following induction. Some treatments included 2.5 mM sodium decanoate addition at 3 hours following induction. Samples for monatin and dry cell weight determination were taken at 24, 30 and 48 hours.

Table 17.1

Monatin per dry cell weight excreted by *E. coli*

		Monatin / dcw (mg/g)	
	Sodium decanoate (mM)	30 hours	48 hours
E.coli MG1655 :: *aspC proA* pProNdedel, pUC19 (control)	0	2.59	2.94
E.coli MG1655 :: *aspC proA* pProNdedel, *ramA* pUC19	0	4.31	5.92

n=2 for all treatments

[0173] Overexpression of RamA without sodium decanoate treatment resulted in greater monatin excretion at both 30 and 48 hours. Monatin per dry cell weight was 5.92 mg/g on average at 48 hours when RamA was overexpressed compared to 2.94 mg/g average with no RamA overexpression. RamA overexpression resulted in a 2-fold increase in monatin excretion. This is evidence that RamA overexpression had a positive impact on the *mar* operon or multiple drug resistance transporter genes or both resulting in increased monatin excretion.

Table 17.2

Monatin per dry cell weight excreted by *E. coli*

		Monatin / dcw (mg/g)	
	Sodium decanoate (mM)	30 hours	48 hours
E.coli MG1655 :: *aspC proA* pProNdedel pUC19 (control)	2.5	4.66	4.31
E.coli MG1655 :: *aspC proA* pProNdedel, *ramA* pUC19	2.5	3.69	13.90

n=2 for all treatments

[0174] Overexpression of RamA with 2.5 mM sodium decanoate treatment resulted in greater monatin excretion at both 30 and 48 hours. Monatin per dry cell weight was 13.90 mg/g on average at 48 hours when RamA was overexpressed compared to 4.31 mg/g average with no RamA overexpression. RamA overexpression in the presence of sodium decanoate resulted in a 3.2-fold increase in monatin excretion. This is evidence that RamA overexpression acted on the *mar* operon or multiple drug resistance transporter genes or both to increase monatin excretion in the presence of sodium decanoate.

Table 17.3

Monatin per dry cell weight excreted by *E. coli ΔacrAB*

	Monatin / dcw (mg/g)		
	24 hours	30 hours	48 hours
E.coli MG1655 ΔAcrAB :: *aspC proA* pProNde del, pUC19 (control)	10.7	29.7	39.3
E.coli MG1655 ΔAcrAB :: *aspC proA* pProNde del, *ramA* pUC19	32.3	41.7	61.1

n=2 for all treatments
2.5 mM sodium decanoate added to all treatments

[0175] Overexpression of RamA in the *E. coli* ΔAcrAB with the monatin operon resulted in greater monatin excretion than the same strain without overexpression of RamA. At 48 hours, overexpression of RamA resulted in 61.1 mg of monatin per gram of dry cell weight and 39.3 mg/g without overexpression of RamA. This represented a 1.5-fold increase in monatin efflux with RamA overexpression. These data are evidence that RamA overexpression increased monatin excretion without involvement of the AcrAB transporter. This positive impact on monatin efflux could be attributed to the activation of the *mar* operon or transporter genes/systems other than AcrAB.

[0176] Thus, we have demonstrated that overexpression of the *ramA* gene in *E. coli* resulted in an increase in monatin efflux. The increase in monatin efflux was also observed in a host background in which the AcrAB system was deleted. This was taken to be evidence that the positive impact of RamA on monatin efflux is also due to its impact on transport systems besides the AcrAB transport system.

EXAMPLE 18

Increase of Monatin Excretion with Overexpression of the MarA Protein

[0177] Transcriptional activation of *acrAB* expression is the main cause of multidrug resistance in strains that overexpress MarA, a member of the AraC family of transcriptional activators. MarA, activates its own transcription and that of a large number of mar regulon genes by binding to DNA regions called marboxes that are located near the promoters for various target genes. The *acrAB* promoter is also adjacent to a marbox at which MarA has been demonstrated to bind and activate transcription. Alekshun, M.N., and Levy, S.B., "Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon," *Antimicrob. Agents Chemother. 41*:2067-2075, (1997).

[0178] In addition, overexpression of MarA has also been demonstrated to result in increased synthesis of the TolC component of the AcrAB-TolC pump complex, which, in combination with the identification of a putative *mar/rob/sox*-box upstream of the *tolC* gene, strongly suggests that *tolC* also

belongs to the mar regulon. Aono, R. *et al.*, "Involvement of outer membrane protein TolC, a possible member of the mar-sox regulon, in maintenance and improvement of organic solvent tolerance of Escherichia coli K-12," *J. Bacteriol. 180*:938-944, (1998).

[0179] The transcriptional activation functions of MarA are reported to be global in nature because MarA can promote the transcription of genes encoding proteins of diverse functions, both in vivo and in vitro. Gene array analysis of a strain constitutively expressing MarA has indicated that more than 60 *E. coli* genes are differentially regulated by this protein (Barbosa, T.M., and Levy, S.B., "Differential expression of over 60 chromosomal genes in *Escherichia coli* by constitutive expression of MarA," *J. Bacteriol. 182*:3467-3474, (2000)), whereas a second study employing an inducible MarA expression system identified an additional 67 MarA-regulated genes. Pomposiello, P.J. *et al.*, "Genome-wide transcriptional profiling of the Escherichia coli responses to superoxide stress and sodium salicylate," *J. Bacteriol. 183*:3890-3902, (2001). It has been reported that MarA is also capable of activating a gene that possess a marbox which diverges substantially from the consensus sequence. Barbosa, T. M. and S. B. Levy, "Activation of the *Escherichia coli nfnB* gene by MarA through a highly divergent marbox in a class II promoter," *Mol. Microbiol. 45*:191-202, (2002).

[0180] Overall, MarA activates expression of the mar regulon, including *acrAB, tolC*, and *marRAB*, whereas MarR acts to downregulate this response by repressing the synthesis of MarA. Although overexpression of MarA from a plasmid is sufficient to activate the mar regulon genes, the addition of the antibiotics tetracycline and chloramphenicol, weak aromatic acids, such as salicylate, and a structurally diverse range of other compounds, such as the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone and the redox-cycling compounds menadione and plumbagin, have all been shown to cause induction of mar regulon expression. Grkovic S *et al.*, "Regulation of bacterial drug export systems," *Microbiol Mol Biol Rev. 66*:671-701, (2002).

[0181] The strain used for the experiment was *E. coli* MG1655:: *aspC / proA /* pProNde together with the *marA* gene from *E.coli* cloned into the pUC19 vector (GenBank/EMBL accession number L09137). The control strain was *E. coli* MG1655:: *aspC / proA /* pProNde along with the pUC19 vector. The

marA gene was amplified from *E.coli* W3110 with PCR technology (known to one skilled in the art) using the primers E.coliMarASalIF – 5'TTAAGGCCGTCGACATGACGATGTCCAGACGCAATA3' and E.coliMarABamHIR – 5'GCAGTGCCGGATCCCTAGCTGTTGTAATGATTTA3'. For inoculum, the *E. coli* strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 100 μg/mL ampicillin and 50 μg/mL kanamycin.

[0182] For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman, *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746, (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ –$6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0183] For treatments, 3.2-3.6 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 100 μg/mL ampicillin and 50 μg/mL kanamycin. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then, 30 °C following induction. At an OD_{600nm} between 0.35-0.50, induction of the plasmid genes was initiated. At induction, 1.0 mM IPTG, 0.5% L-arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mLs of Balch's vitamins were added. Additions of 10 g/L L-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3.0 hours following induction. At 24 hours

after inoculation, 10 g/L of sodium pyruvate was added to each flask again. Some treatments included 2.5 mM sodium decanoate addition at 3.0 hours following induction. Samples for monatin and dry cell weight determination were taken at 24, 30 and 72 hours.

Table 18.1

Monatin per dry cell weight excreted by *E.coli*

		Mean monatin per dry cell weight (mg/g)	
Strain	Treatment (sodium decanoate addition in mM)	30 hours	72 hours
E.coli MG1655 aspC proA pProNde del, pUC19 (control)	0	2.2	2.3
E.coli MG1655 aspC proA pProNde del, MarA pUC19	0	2.7	23.4

n=2 for all treatments

[0184] Overexpression of *marA* results in increased excretion of monatin (monatin per dry cell weight) in the absence of sodium decanoate addition as seen above. There is a 10 fold increase in mg monatin per g dry cell weight due to overexpression of the marA gene. This significant increase in monatin efflux can be explained by the impact of MarA on the AcrAB transport system, the increased transcription of the *tolC* gene, which interacts with multiple transport systems, and/or the possible upregulation of other transporters that could play a role in monatin efflux.

Table 18.2

Monatin per dry cell weight excreted by *E.coli*

Strain	Treatment (sodium decanoate addition in mM)	Mean monatin per dry cell weight (mg/g) 30 hours	72 hours
E.coli MG1655 aspC proA pProNde , del pUC19 (control)	2.5	2.4	2.7
E.coli MG1655 AspC ProA pProNde ,del MarA pUC19	2.5	8.5	51.9

n=2 for all treatments

[0185] Overexpression of *marA* results in additional increased excretion of monatin (monatin per dry cell weight) in the presence of sodium decanoate addition as seen above. With 2.5 mM of sodium decanoate addition, the *marA* strain excreted 51.9 mg monatin per dry cell weight on average, and the control strain was only 2.7 mg/g at 72 hours, this represents a 19 fold increase in monatin efflux. This reveals the substantial effect that *marA* overexpression has on transporter(s) that excrete monatin. There appears to be a synergistic effect of sodium decanoate with *marA* overexpression.

Table 18.4

Monatin per dry cell weight excreted by *E.coli* Δ*acrAB* with the monatin operon

Strain	Strain / insert	Mean monatin per dry cell weight (mg/g) 24	30	72
E.coli MG1655 ΔAcrAB AspC ProA pProNde del pUC19 (control)	ΔAcrAB	22.2	20.6	62.6
E.coli MG1655 ΔAcrAB AspC ProA pProNde del MarA pUC19	ΔAcrAB with *marA* insert	54.6	56.0	209.0

n=2 for all treatments
2.5 mM sodium decanoate addition for all treatments

[0186] The combination of *marA* overexpression with the *acrAB* knockout mutant resulted in an average of 209 mg of monatin per gram dry cell weight at 72 hours versus 62.6 for the acrAB knockout mutant without the *marA* plasmid. Even by 24 hours, the *acrAB* knockout / *marA* overexpression strain outperformed the control by over two-fold (54.6 to 22.2 mg monatin / g dcw). This is an example of the combination of a deletion of the AcrAB transporter and overexpression of a regulatory gene, for example *marA*, that has a synergistic effect on monatin excretion.

[0187] MarA is known to activate *acrAB* expression, but in the *acrAB* knockout, even more monatin was excreted than usual. It has been reported that MarA expression can increase the expression of a number of genes involved in transport, in addition to *acrA* such as mtr (tryptophan-specific transport protein), ompX (outer membrane protein X), and yadG (putative ATP binding component of a transport system). Thus, this effect may be attributed to the induction of a transporter other than AcrAB, involved in monatin excretion. It is also known that MarA activates the transcription of *tolC*, which is a component of multiple transporter systems. Thus an increase in monatin transport could be attributed to the action of multiple transporters requiring TolC.

[0188] These results demonstrate that MarA has a strong positive influence on monatin efflux in *Escherichia coli*.

EXAMPLE 19

Increase of Monatin Excretion with Overexpression of the BaeR Protein

[0189] The BaeSR two-component regulatory system controls expression of exporter genes, conferring drug resistance in *Escherichia coli*. Nagakubo, S. *et al.*, *J. Bacteriol. 184*:4161-4167, (2002); Baranova, N. and Nikaido, H., "The *baeSR* two-component regulatory system activates transcription of the *yegMNOB* (*mdtABCD*) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate," *J. Bacteriol. 184*:4168-4176, (2002). It has been reported that the BaeSR two-component system modulates the drug resistance of *E. coli* by regulating the expression of

drug transporter genes. Baranova, N. and Nikaido, H., "The *baeSR* two-component regulatory system activates transcription of the *yegMNOB* (*mdtABCD*) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate," *J. Bacteriol. 184*:4168-4176, (2002); Nagakubo, S.K., *et al.*, "The putative response regulator BaeR stimulates multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC," *J. Bacteriol. 184*:4161-4167, (2002). The response regulator BaeR modulates the expression of *mdtABC* and *acrD*, which encode multidurug exporter systems. Hirakawa, H.K., *et al.* "Comprehensive studies on the drug resistance mediated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*," *J. Bacteriol. 185*:1851-1856, (2003); Hirakawa, H.K., *et al.*, "ß-Lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*," *J. Antimicrob. Chemother. 52*:576-582, (2003). Overproduction of BaeR, in the background of a deficiency of the *E. coli* major multidrug exporter AcrB, reportedly confers resistance against ß-lactams, novobiocin, sodium dodecyl sulfate, and bile salts. It is also reported that BaeR increased the expression of the outer membrane channel *tolC* gene, which is required for the function of the MdtABC, AcrD and other transport systems. Nishino, K., *et al.*, "Roles of TolC-dependent multidrug transporters of *Escherichia coli* in resistance to ß-lactams," *Antimicrob. Agents Chemother. 47*:3030-3033, (2003); Nishino, K., and Yamaguchi, A., "Analysis of a complete library of putative drug transporter genes in *Escherichia coli*," *J. Bacteriol. 183*:5803-5812, (2001).

[0190] The strains used for the experiment were *E. coli* MG1655:: *aspC* / *proA* / pProNde in combination with either *baeR* or no insert in the pUC19 vector. The baeR gene was amplified from *E.coli* W3110 by using primers, E.colibaeRSalIF 5'GGCCTTCCGTCGACATGACCGAGTTACCAATC3' and E.colibaeRBamHIR 5'TTCCAAGGTTGGATCCCTAAACGATGCGGCAGGC3', which introduced SalI and BamHI sites at the end of the amplified fragment. The PCR fragment was cloned between the *SalI* and *BamHI* sites of the vector pUC19 (GenBank/EMBL accession number L09137). For inoculum, the *E.*

coli strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 100 μg/mL ampicillin and 50 μg/mL kanamycin.

[0191] For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746 (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ $-6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0192] For treatments, 3.5-5.0 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 100 μg/mL ampicillin and 50 μg/mL kanamycin. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then, 30 °C following induction. At 0.35-0.38 OD_{600nm} , induction of the plasmid genes was initiated. At induction, 1.0 mM IPTG, 0.5% L-arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mLs of Balch's vitamins were added. Additions of 10 g/L L-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3.5 hours following induction. At 24 hours after inoculation, another 10 g/L increment of sodium pyruvate was added to each flask. Some treatments included 2.5 mM sodium decanoate addition at 3.5 hours following induction. Samples for monatin and dry cell weight determination were taken at 24, 30 and 72 hours.

Table 19.1

Monatin per dry cell weight excreted by *E. coli*

Strain	Treatment (sodium decanoate addition in mM)	Mean monatin per dry cell weight (mg/g)	
		30 hours	72 hours
E.coli MG1655 AspC ProA pProNde del pUC19	0	2.2	2.3
E.coli MG1655 AspC ProA pProNde del BaeR pUC19	0	2.5	24.9

n=2 for all treatments

[0193] Overexpression of *baeR* resulted in greater monatin excretion with no sodium decanoate when compared to the control strain without *baeR* overexpression. At 72 hours, the *baeR* overexpression strain accumulated averages of 24.9 mg monatin per gram dry cell weight compared to 2.3 for the non-*baeR* control strain under similar conditions. This demonstrates that *baeR* overexpression without decanoate treatment results in approximately an 11-fold increase in monatin excretion. This effect could be due to activation of *mdtABCD* or *acrD* or both transport systems. The native AcrAB system might also play a role in monatin transport. In addition, it is known that BaeR can increase *tolC* gene expression which is required for the function of several export systems.

Table 19.2

Monatin per dry cell weight excreted by *E. coli*

		Mean monatin per dry cell weight (mg/g)	
Strain	Treatment (sodium decanoate addition in mM)	30 hours	72 hours
E.coli MG1655 AspC ProA pProNde del pUC19	2.5	2.4	2.7
E.coli MG1655 AspC ProA pProNde del BaeR pUC19	2.5	16.2	66.8

n=2 for all treatments

[0194] Overexpression of *baeR* resulted in greater monatin excretion with 2.5 mM sodium decanoate when compared to the control strain without *baeR* overexpression. At 72 hours, the *baeR* overexpression strain accumulated averages of 66.8 mg monatin per gram dry cell weight compared to 2.7 for the non-*baeR* strain under similar conditions. This demonstrates that *baeR* overexpression in combination with decanoate treatment results in approximately 25-fold increased monatin excretion when compared to the treatment without decanoate. It is known that sodium decanoate can activate the AcrAB transport system. In addition, it is known that BaeR can increase *tolC* gene expression which is required for the function of several export systems. This enhanced monatin efflux may be due to activation of *mdtABCD* and/or *acrD* in addition to the AcrAB transport system activation due to decanoate addition.

Table 19.3

Monatin per dry cell weight excreted by *E. coli*

	Mean monatin per dry cell weight (mg/g)		
Strain	24 hours	30 hours	48 hours
E.coli MG1655 ΔacrAB AspC ProA pProNde del pUC19 (control)	10.7	29.7	39.3
E.coli MG1655 ΔacrAB AspC ProA pProNde del BaeR pUC19	34.4	85.0	137.3

n=2 for all treatments
2.5 mM sodium decanoate addition for all treatments

[0195] It has been reported that the native multi-drug exporter AcrB can mask the effect of *baeR* overexpression. In order to determine the role of *baeR* overexpression in the absence of the AcrAB transport system, a host strain with a deletion of the *acrAB* genes was used. In a Δ*acrAB* host strain, overexpression of the *baeR* gene resulted in a 3.5-fold increase in monatin excretion (137.3 to 39.3 mg monatin per g dcw) over the control Δ*acrAB* strain. This is clear evidence that transporters in addition to AcrAB were involved in monatin transport. In addition, it is known that BaeR can increase *tolC* gene expression which is required for the function of several export systems in addition to the MdtABCD or AcrD multidrug transport systems. Nishino, K., *et al.*, "Genome-wide analysis of *Escherichia coli* gene expression responsive to the BaeSR two-component regulatory system," *J Bacteriol. 187*:1763-1772, (March 2005). Thus, either MdtABCD or AcrD or both transporter systems were involved in monatin excretion in the *acrAB* knockout strain.

[0196] Thus, we have conclusively demonstrated the positive impact that BaeR has on monatin efflux. It has been reported that indole induces *mdtABCD* and *acrD* gene expression via the BaeSR two-component signal transduction system. Nishino, K., *et al.*, "Genome-wide analysis of

Escherichia coli gene expression responsive to the BaeSR two-component regulatory system," *J Bacteriol. 187*:1763-1772, (March 2005). It is reasonable to expect that indole treatment should also have a positive impact on monatin efflux. The quorum sensing regulator SdiA is reported to control the expression of acrAB and acrD. Wei, Y., *et al.*, "Global impact of *sdiA* amplification revealed by comprehensive gene expression profiling of *Escherichia coli*," *J. Bacteriol. 183*:2265-2272, (2001). It is also reasonable then to expect that SdiA could have a positive impact on monatin efflux.

EXAMPLE 20

Increase of Monatin Excretion in *Corynebacterium glutamicum* with Increased Temperature and Sodium Pyruvate Treatments

[0197] *C. glutamicum* 13032 strains transformed with *aspCProA* pEKEX-2 were grown overnight in LB medium supplemented with 25 μg/mL kanamycin incubated at 30 °C and shaking at 250 rpm. For the experimental treatment flasks, 100 mL of Kraemer's A medium was used in each shake flask. Hoisted C., and Kraemer, R. "Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*," *Arch. Microbiol 151*:342-347, (1989). Kraemer's A medium contained (per liter): 5 g $(NH_4)_2SO_4$, 5 g urea, 2g KH_2PO_4 , 1.53 K_2HPO_4, 0.249 g $MgSO_4*7H_2O$, 50 g glucose, 0.01 g $FeSO_4$-$7H_2O$, 0.01 g $MnSO_4$-H_2O, 0.01 g $CaCl_2$-$2H_2O$, 0.03 mg $ZnSO_4$-$7H_2O$, 0.1mg H_3BO_3, 0.07 mg $CaCl_2$-$6H_2O$, 0.01 mg $NiCl_2$-$2H_2O$, 0.03 mg $CuCl_2$-$2H_2O$, 0.1 mg as Mo^{+6} from $(NH_4)_6Mo_7O_{24}$-$4H_2O$ and 1 μg biotin. The pH was adjusted to 7.0.

[0198] For treatments, 3.5-5.0 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks. Conditions for treatments included 30° or 35° C throughout the experiment and 250 rpm agitation. At an OD_{600nm}, between 0.45-0.51, induction of the monatin operon genes was initiated. IPTG at 1.0 mM was used for induction, and additions at the time of induction included 0.2 mLs of Balch's 1000X vitamin stock and 0.5 mM pyridoxine hydrochloride. Additions of 1.0 g L-tryptophan, 10 or 15 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP"), were made 3 hours and also at 24 hours following induction. A total of 20 or 30 g/L

sodium pyruvate was added to each flask by adding 10 or 15 g/l at each of the two feeding times. Samples for monatin and dry cell weight determination were taken at 24, 30 and 48 hours.

Table 20.1

Increasing temperature and pyruvate resulted in increased monatin efflux per dry cell weight

	Monatin / dry cell weight (mg/g)			
Treatment	24 hours	31 hours	48 hours	54 hours
30 °C, 20 g/L pyruvate	0.2	2.1	2.7	2.7
30 °C, 30 g/L pyruvate	0.3	1.8	4.3	4.7
35 °C, 20 g/L pyruvate	5.4	14.7	23.5	23.8
35 °C, 30 g/L pyruvate	5.8	18.1	29.4	35.2

[0199] At 30 °C, increased levels of sodium pyruvate resulted in increased monatin per dry cell weight (4.7 mg/g vs. 2.7 at 54 hours). At 35 °C, additional pyruvate increased the monatin per dry cell weight even further from 23.8 to 35.2 mg /g. An increase of 5 °C resulted in a 7 to 9 fold increase in monatin per dry cell weight. Increased temperature from 30 to 35 °C also reduced dry cell weight from 7.48 g/L to 2.50 g/L. Both temperature and sodium pyruvate were highly statistically significant factors for monatin per dry cell weight in the factorial design experiment with 16 total treatments. The increased yield of monatin with a reduction in biomass suggests that a change in carbon flux distribution occurred. Ohnishi, J., *et al.*, *Appl Microbiol Biotechnol 62*:69-75, (2003).

[0200] Thus we have demonstrated that through manipulation of the amount of sodium pyruvate added and the incubation temperature of the organism, monatin efflux can be increased. Presumably this increased efflux in monatin is accompanied by an increased in monatin production, but the impact of factors on monatin efflux itself cannot be ruled out. The continued use of statistical design of experiments, known in the art, to vary growth media composition and growth conditions, can be used to increase the amount of monatin produced and effluxed out of the cell. Further benefits in monatin efflux may be obtained through the use of ethambutol in combination with, but

not limited to, one or more of the following reagents that are known to affect the mycolic acid layer of *Coryenbacterium*, including Tween, biotin, and/or ampicillin.

EXAMPLE 21

Increase of Monatin Excretion in *Corynebacterium glutamicum* with Biotin and Ampicillin Treatments

[0201] *C. glutamicum* ATCC 13032 strains transformed with *aspCProA* pEKEX-2 were grown overnight in LB medium supplemented with 50 μg/mL kanamycin incubated at 30 °C and shaking at 250 rpm. For the experimental treatment flasks, 100 mL of Kraemer's A medium was used in each shake flask. Hoisted C., and Kraemer, R. "Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*," *Arch. Microbiol 151*:342-347, (1989). Kraemer's A medium contained (per liter): 5 g $(NH_4)_2SO_4$, 5 g urea, 2g KH_2PO_4 , 1.53 K_2HPO_4, 0.249 g $MgSO_4*7H_2O$, 50 g glucose, 0.01 g $FeSO_4$-$7H_2O$, 0.01 g $MnSO_4$-H_2O, 0.01 g $CaCl_2$-$2H_2O$, 0.03 mg $ZnSO_4$-$7H_2O$, 0.1mg H_3BO_3, 0.07 mg $CaCl_2$-$6H_2O$, 0.01 mg $NiCl_2$-$2H_2O$, 0.03 mg $CuCl_2$-$2H_2O$, 0.1 mg as Mo^{+6} from $(NH_4)_6Mo_7O_{24}$-$4H_2O$ and 1 μg or 200 μg biotin. The pH was adjusted to 7.0.

[0202] For treatments, inoculum was added to 0.100 absorbance (600 nm) in 100 mL medium volume in 500 mL baffled shake flasks. Conditions for treatments included 37 °C throughout the experiment and 250 rpm agitation. At 0.26-0.33 OD_{600nm}, induction of the monatin operon genes was initiated. IPTG at 0.5 mM was used for induction and 1.0 mM pyridoxine hydrochloride and 0.04 mM pyridoxal-5'-phosphate ("PLP") were also added at the time of induction. Additions of 1.0 g L-tryptophan and 5 g/L sodium pyruvate and ampicillin (either 0 or 10 μg/ml) were made 3 hours following induction. Samples for monatin and dry cell weight determination were taken at 24 and 48 hours.

Table 21.1

Addition of ampicillin and reduction of biotin resulted in increased monatin efflux per dry cell weight

			Monatin / dry cell weight (mg/g)	
Treatment combination	Ampicillin (μg/mL)	Biotin (μg/L)	24 hours	48 hours
1	0	200	0.9	1.7
2	0	1	1.9*	4.5
3	10	200	12.1	9.3
4	10	1	16.2	13.5

n= 2 or 3 depending on treatment combination
* n=1

[0203] Addition of ampicillin at 3 hours following induction resulted in a 3 to 12 fold increase in monatin per dry cell weight. Reduction in biotin from 200 to 1 μg/L in the initial medium resulted in an increase in monatin per dry cell weight by 1.0 to 4.2 mg/g. The treatment combination of ampicillin and biotin (10 μg/mL and 1 μg/L, respectively) increased monatin per dry cell weight by 7.9 to 18 fold (13.5 / 1.7 and 16.2 / 0.9).

[0204] Thus we have demonstrated that through manipulation of the amount of biotin and ampicillin that the host organism is exposed to, monatin efflux can be increased. Presumably this increased efflux in monatin is accompanied by an increase in monatin production, but the direct impact of biotin and/or ampicillin on monatin efflux itself cannot be ruled out.

[0205] The continued use of statistical design of experiments, known in the art, to vary growth media composition and growth conditions, can be used to increase the amount of monatin produced and effluxed out of the cell. Further benefits in monatin efflux may be obtained through the use of ethambutol in combination with, but not limited to, one or more reagents that are known to affect the mycolic acid layer of *Corynebacterium*, including Tween, biotin, and/or ampicillin. Eggeling, L. and Sahm, H., "The Cell Wall Barrier of *Corynebacterium glutamicum* and Amino Acid Efflux," *J. BioSci. and BioEng. 92*:201-213, (2001).

EXAMPLE 22

Increase of Monatin Excretion in *Corynebacterium* Using Ethambutol

[0206] It was reported that the addition of ethambutol ("EMB") to growing cultures of *C. glutamicum* resulted in L-glutamate efflux whereas in the absence of EMB, no efflux occurred. Radmacher, E., *et al.*, "Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*," *Microbiology 151*:1359-1368, (May 2005). EMB reportedly targets a series of arabinosyltransferases (EmbCAB) at the molecular level. A single arabinosyltransferase-encoding *emb* gene of *C. glutamicum* was placed under the control of a Tet repressor ("TetR"). Experiments with this strain, as well as with an emb-overexpressing strain, coupled with biochemical analyses showed that emb expression was correlated with L-glutamate efflux, and increased EMB resistance. In addition EMB caused less arabinan deposition in cell wall arabinogalactan, and a reduced content of cell-wall-bound mycolic acids. Thus, EMB addition resulted in a marked disordering of the cell envelope, which was also borne out through examination of the cellular morphology.

[0207] Because it is known that an altered lipid composition of the plasma membrane of *C. glutamicum* can result in L-glutamate efflux, it has been speculated that major structural alterations of the cell envelope are transmitted to the membrane, which in turn activates an export system, perhaps via increased membrane tension. Radmacher, E., *et al.*, "Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*," *Microbiology 151*:1359-1368, (May 2005).

[0208] *C. glutamicum* ATCC 13032 strains transformed with *aspCProA*pEKEX-2 were grown overnight in LB medium supplemented with 25 μg/mL kanamycin incubated at 30°C and shaking at 250 rpm. For the experimental treatment flasks, 100 mL of Kraemer's A medium was used in each shake flask. Hoisted C,, and Kraemer, R. "Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*," *Arch. Microbiol 151*:342-347, (1989). Kraemer's A medium contained (per liter): 5 g $(NH_4)_2SO_4$, 5 g urea, 2g KH_2PO_4 , 1.53 K_2HPO_4,

0.249 g $MgSO_4*7H_2O$, 50 g glucose, 0.01 g $FeSO_4$-$7H_2O$, 0.01 g $MnSO_4$-H_2O, 0.01 g $CaCl_2$-$2H_2O$, 0.03 mg $ZnSO_4$-$7H_2O$, 0.1mg H_3BO_3, 0.07 mg $CaCl_2$-$6H_2O$, 0.01 mg $NiCl_2$-$2H_2O$, 0.03 mg $CuCl_2$-$2H_2O$, 0.1 mg as Mo^{+6} from $(NH_4)_6Mo_7O_{24}$-$4H_2O$ and 1 μg biotin. The pH was adjusted to 7.0.

[0209] For treatments, 3.5-5.0 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks. Conditions for the treatments included 30 °C throughout the experiment and 250 rpm agitation. Ethambutol (10 mg/L) was added either prior to inoculation of shake flasks or at the first feeding time when tryptophan and pyruvate were added. At 0.40-0.51 OD_{600nm}, induction of the monatin operon genes was initiated. IPTG at 1.0 mM was used for induction, and additions at the time of induction included 0.2 mLs of Balch's vitamins and 0.5 mM pyridoxine hydrochloride. Additions of 1 g L-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP"), were made 3 hours following induction. Samples for monatin and dry cell weight determination were taken at 24, 30/31 and 48 hours.

Table 22.1

Ethambutol addition increased monatin per dry cell weight

		Monatin / dry cell weight (mg/g)		
Strain	ethambutol (mg/L)	24 hours	31 hours	48 hours
C.glutamicum 13032:: *aspC / proA* / pEKEX2-2	0	0.43	0.47	0.51
C.glutamicum 13032:: *aspC / proA* / pEKEX2-2	10	1.16	1.15	1.06

n=2 for all treatments

[0210] Monatin excretion (mg/g dry cell weight) increased with ethambutol addition. At 48 hours, the 10 mg/l ethambutol treatment resulted in an average of 1.06 mg of monatin per gram dry cell weight, while the control without ethambutol excreted 0.51 mg of monatin per gram dry cell weight on average.

Table 22.2

Ethambutol addition with additional sodium pyruvate increased monatin per dry cell weight

Strain	ethambutol (mg/l)	Monatin / dry cell weight (mg/g)		
		24 hours	31 hours	48 hours
C.glutamicum 13032:: AspC / ProA / pEKEX2-2	0	0.43	0.47	0.51
C.glutamicum 13032:: AspC / ProA / pEKEX2-2	10	1.04	1.53	2.19

n=2 for all treatments

[0211] With another 10 g/L sodium pyruvate dosage at 24 hours following inoculation, a further increase in monatin production / excretion was observed. At 48 hours, the 10 mg/L ethambutol treatment with the additional sodium pyruvate supplementation, had 2.19 mg / g of monatin per dry cell weight on average versus 1.06 without additional pyruvate at 24 hours for the same ethambutol addition.

Table 22.3

Ethambutol addition combined with Tween / ampicillin treatment further increased monatin per dry cell weight

Strain	ethambutol (mg/L)	Monatin / dry cell weight (mg/g)		
		24 hours	30 hours	48 hours
C.glutamicum 13032:: AP pEKEX2-2	0	0.30	0.33	0.74
C.glutamicum 13032:: AP pEKEX2-2	10	6.94	8.80	9.14

n=2 for all treatments

[0212] With addition of 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) and 10 μg/ml ampicillin at feeding time (3 hours following induction time) combined with ethambutol treatment, monatin excretion was observed to increase even more. There was a synergistic effect between Tween, ampicillin and ethambutol treatment that was favorable for monatin efflux. The Tween / ampicillin treatment with 10 mg/l ethambutol resulted in 12-fold greater monatin excreted per dry cell weight than the same treatment

without ethambutol addition (9.14 mg monatin/g dry cell weight versus 0.74 monatin/g dry cell weight, respectively).

[0213] Thus we conclusively demonstrated that ethambutol addition has a positive impact on monatin efflux in *Corynebacterium*. These data also suggest that monatin may be excreted via the same transporter as glutamate because it is reported that ethambutol affects only glutamate excretion and not excretion of other amino acids. Radmacher E., *et al.*, "Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*," *Microbiology, 151*:1359-68, (May 2005). In addition, we also demonstrated that the positive effect of ethambutol treatment combined, with Tween and ampicillin, results in an amplified monatin efflux response in *Corynebacterium* that is greater than that observed with ethambutol alone.

EXAMPLE 23

Increase of monatin excretion in a host strain with a *cysH* deletion

[0214] *CysH* (Phosphoadenylyl sulfate ("PAPS") reductase), *icdA* (isocitrate dehydrogenase), *metE*, or *purB* (adenylosuccinate lyase) mutations have been reported to cause an activation of the AcrAB transport system. The metabolites that accumulate upstream of the blocks caused by the mutations could increase the levels of the AcrAB-TolC pump. Helling R.B., *et al.*, "Toxic waste disposal in *Escherichia coli*," *J Bacteriol. 184*:3699-3703, (2002). We tested the impact that a cysH deletion had on monatin efflux.

[0215] Primers were designed to create the desired knockout in *E. coli* BL21DE3 by PCR from template pKD4 as described. Datsenko, K.A., and Wanner, B.L., "One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products," *Proceed. Natl. Acad. Sci. USA 97*:6640-6645, (2000).

[0216] cysH knockout primer sequences:

E. coli cysHKO-Forward

5'CGCGTGAGCGTCGCATCAGGCAAGGCAAACAGTGAGGAATCTAT GTCCAAAGTGTAGGCTGGAGCTGCTTC 3'

E. coli cysHKO-reverse

5'CGCCCCCATCATTTCTGACAGAGGCGTTTAATTTGTCCGGCAATA TTTACCCTTCCATATGAATATCCTCCTTAG3'

[0217] The PCR products for deletion of *cysH* were amplified using the following PCR protocol. In a 100 μL reaction, 1 μL template (pKD4) (Datsenko, K.A., and Wanner, B.L., "One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products," *Proceed. Natl. Acad. Sci. USA 97*:6640-6645, (2000)), 0.4 μM of each primer, 0.4 mM each dNTP, 1X PCR buffer, and 2 μL Pfu Turbo Polymerase (Stratagene, La Jolla, CA) were used. The thermocycler program used included a hot start at 94 °C for 30 seconds, 30 repetitions of the following steps: 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 4 minutes. After the 30 repetitions the sample was maintained at 72°C for 10 minutes and then stored at 4 °C. This PCR protocol produced a product of 1.6 Kb.

[0218] The PCR product was purified using the Qiagen PCR Cleanup kit (Valencia, CA). The PCR product was quantified using a SmartSpec 3000™ spectrophotometer.

[0219] The purified PCR product was used to transform *E. coli* strain BW25113/pKD46. Datsenko, K.A., and Wanner, B.L., "One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products, *Proceed. Natl. Acad. Sci. USA 97*:6640-6645 (2000). 1 μL of the PCR product was added to 40 μL of cells, which were transformed by electroporation using the BioRad Gene Pulsar II under the following conditions: 2.5 kV, 25 μF, 200 ohm in a 0.2 cm cuvette. The cells were allowed to recover in 500 μL of SOC for 3 hours at 37 °C with shaking at 225 rpm. Cells were plated on LB plates containing kanamycin (50 μg/mL). The plates were incubated at 37 °C overnight. Five kanamycin-resistant transformants were colony PCR-screened to confirm product.

[0220] **Lysate production**: P1 phage lysate was made for the BW25113Δ*cysH* strain, to allow transfer of the knockout into the *E. coli* BL21DE3 production host. The donor strain was grown overnight in LB medium containing 25 μg/ mL kanamycin. The culture was used to inoculate

fresh LB medium containing 5 mM $CaCl_2$ using a 1:10 dilution and were incubated for 70 minutes at 37 °C. One mL of culture was incubated with 3 μL or 5 μL of a phage stock (ATCC 25404-B1) at 37 °C for 20 minutes. The phage/culture was then mixed with 4 mL of soft agar containing 5 mM $CaCl_2$ and overlaid on LB medium. Control experiments were set up using no phage. The plates were incubated at 37 °C, right-side up, for 5 hours, after which confluent lysis was observed for all plates containing phage; the control plates had cell lawns as expected. The plates were incubated overnight at 37 °C, after which phage-resistant colonies were observed on experimental plates as expected. The soft agar from each plate was scraped into a centrifuge tube using a sterile disposable loop. Two mL of LB was used to rinse the plate, and the rinse was combined with the soft agar in the centrifuge tube. Five drops of chloroform were added to the tubes, which were gently mixed and incubated at room temperature for 20 minutes. The mixtures were centrifuged at 10,000 x g for 10 minutes and the supernatants were filtered with a 0.2 μm syringe filter to obtain phage lysates. The phage lysate was stored at 4 °C.

[0221] **Transduction into production host:** The *cysH* knockout was transferred to strain *E. coli* BL21DE3 by P1 transduction to generate strain BL21DE3Δ*cysH*. *E. coli* BL21DE3Δ*cysH* was grown overnight in LB medium containing 25 μg/mL chloramphenicol. The culture was used to inoculate 5 mL of fresh LB medium, supplemented with 5 mM $CaCl_2$ using a 1:10 dilution. The subculture was incubated for 60 minutes at 37 °C. The culture was centrifuged, resuspended in 500 μL of MC buffer (0.1 M MgS04, 5 mM $CaCl_2$), and incubated at room temperature for 20 minutes. Various dilutions of the donor lysate (1:100 to 1X in MC buffer) were added in equal volume to 100 μL of culture. The mixtures were incubated for 20 minutes at 37 °C, after which 200 μL of citrate buffer (0.1 M citric acid and 220 mM NaOH adjusted to pH 5.5) and one mL of LB were added to each tube. The cultures were incubated at 37 °C for one hour with agitation at 200 rpm, followed by centrifugation to obtain a cell pellet. The cell pellets were resuspended in 100 μL of citrate buffer and plated on LB medium containing 25 μg/mL kanamycin. Single kanamycin -resistant colonies were purified by restreaking on appropriate selective media.

[0222] Single kanamycin-resistant colonies were tested for cysteine and methionine auxotrophy (the phenotype which would confirm *cysH* deletion) by growth on M9 media supplemented with L-Cysteine and/or L-Methionine.

[0223] For inoculum, the *E. coli* strains BL21 DE3 *aspC proA* pET32 and BL21 DE3 ΔcysH:: *aspC proA* pET32 were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 100 μg/mL ampicillin.

[0224] For the experimental treatments, Trp-1 + glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt, F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746 (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ –$6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2$ – $4H_2O$, and 0.14 g $ZnSO_4$ – $7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0225] For treatments, 3.0-5.0 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 100 μg/mL ampicillin. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then, 30 °C following induction. At an OD_{600nm} between 0.44-0.52, induction of the plasmid genes was initiated. At induction, 1.0 mM IPTG, 0.5 mM pyridoxine hydrochloride, and 0.2 mLs of Balch's vitamins were added. Additions of 10 g/L L-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3.0 hours following induction. At 3 hours after the initial feeding of pyruvate, another 10 g/L of sodium pyruvate was added to each flask. Some treatments included 2.5 mM sodium decanoate

(or sterile, distilled water for the 0 mM decanoate treatment) addition at 3.0 hours following induction. Samples for monatin and dry cell weight determination were taken at 24, 30 and 48 hours.

Table 23.1

Monatin per dry cell weight excreted by *E.coli* with no decanoate addition

		Monatin / dry cell wt. (mg/g)		
Strain	Sodium decanoate (mM)	24 hours	30 hours	48 hours
E. coli BL21 DE3 *aspC proA* pET32	0	4.6	5.2	4.2
E. coli BL21 DE3 □cysH:: *aspC proA* pET32	0	6.8	10.8	21.4

n=2

[0226] Without sodium decanoate addition, the ΔcysH mutant excreted greater than 5 fold more monatin per dry cell weight by 48 hours than the control strain (21.4 vs. 4.2 mg/g). These results demonstrated that the ΔcysH mutant strain excreted more monatin. This effect is most likely due to induction of the AcrAB and/ or other transport systems.

Table 23.2

Monatin per dry cell weight excreted by *E.coli* with addition of sodium decanoate

		Monatin / dry cell wt. (mg/g)		
Strain	Sodium decanoate (mM)	24 hours	30 hours	48 hours
E. coli BL21 DE3 *aspC proA* pET32	2.5	70.9	75.0	44.0
E. coli BL21 DE3 ΔcysH:: *aspC proA* pET32	2.5	115.0	103.1	73.3

n=2

[0227] With the addition of sodium decanoate which is an inducer of the AcrAB transport system, even greater amounts of monatin were excreted in the ΔcysH mutant. The mutant strain excreted greater than 100 mg/g of

monatin per dry cell weight by 24 hours. This is greater than 60% more than the control strain (115.0 vs. 70.9 mg monatin/g dry cell weight). These results confirmed that the ΔcysH mutant strain excreted more monatin. This effect is most likely due to induction of the AcrAB and/ or other transport systems. The fact that maximum monatin efflux was observed with a combination of the ΔcysH mutant and sodium decanoate addition points to the involvement of the AcrAB transport system and/ or other transport systems.

[0228] Because *cysH, icdA* (isocitrate dehydrogenase), *metE*, or *purB* (adenylosuccinate lyase) mutations have all been reported to cause an activation of the AcrAB transport system (Helling, R.B., *et al.*, "Toxic waste disposal in *Escherichia coli*," *J. Bacteriol. 184*:3699-3703, (2002), we would expect an increased monatin efflux in strains with deletions in one or more of the following genes: *cysH, icdA, metE*, or *purB*

EXAMPLE 24

AcrEF transporter system may impact monatin efflux

[0229] *Escherichia coli* produces indole, a metabolite of tryptophan, under certain physiological conditions. Inactivation of the *acrEF* gene, the product of which is an energy-dependent multiple drug efflux pump, was shown to decrease indole excretion. Reintroduction of the *acrEF* gene restored indole excretion. A delta *acrEF* mutant reportedly accumulated more intracellular indole than the parent. This mutant was more susceptible to the growth-inhibitory effect of indole than the parent. These results were taken as evidence that the AcrEF system plays a significant role in indole efflux. Kawamura-Sato, K, *et al.*, "Role of multiple efflux pumps in *Escherichia coli* in indole expulsion," *FEMS Microbiol Lett. 179*:345-352, (1999). Inactivation of the envR gene results in over-expression of the AcrEF transport system.

[0230] Primers were designed to create the *ΔenvR* knockout product by PCR from template pKD3. Datsenko, K.A. and Wanner, B.L., "One-step inactivation of chromosomal genes in *Escherichia coli K-12* using PCR products," *Proceed. Natl. Acad. Sci. USA 97*:6640-6645, (2000).

[0231] The *ΔenvR* knockout product was constructed and amplified using *Pfu Turbo* DNA polymerase (Stratagene, La Jolla, CA) and pKD3 (Datsenko, K.A., and Wanner, B.L., "One-step inactivation of chromosomal genes in *Escherichia coli K-12* using PCR products," *Proceed. Natl. Acad. Sci. USA* 97:6640-6645, (2000)) as a template and primers ENVR1 (5' CACTCTGTGTCGAATATATTTATTTCCTGAATAATTAATCTGGTGTA GGCTGGAGCTGCTTC 3') and ENVR2 (5' ACTGTGACGAACTGAATTTTCAGGACAGAATGTGAATTTACATATG AATATCCTCCTTA 3'). The thermocycler program used included a hot start at 95 °C for 2 minutes, 10 repetitions of 95 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 2 minutes, followed by 25 cycles of incubations at 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 2 minutes. The final step was incubation at 70 °C for 7 minutes. The PCR product from two PCR reactions (200 µL total volume) was purified using the QIAquick Gel Extraction Kit (Qiagen Hilden, Germany) using the manufacturer's instructions. The *ΔenvR* knockout PCR product was eluted with 10 µL of double distilled water.

[0232] Strain BW25113/pKD46 (Datsenko, K.A. and Wanner, B.L., "One-step inactivation of chromosomal genes in *Escherichia coli K-12* using PCR products," Proceed. Natl. Acad. Sci., USA, 97:6640-6645, (2000)) was transformed with 1 µL of PCR product (250 ng) by electroporation using the Gene Pulsar II Electroporation system (Bio-Rad, Hercules, CA) as recommended by the manufacturer and plated on LB solid media with chloramphenicol 10 µg/mL after a 150 minutes of outgrowth in SOC medium (Molecular Cloning, A Laboratory Manual 3rd Edition 2001, Sambrook and Russell, Cold Spring Harbor Laboratory Press, NY USA). The plates were incubated at 37 °C overnight.

[0233] Chloramphenicol resistant colonies were screened by PCR to determine the status of the *envR* locus. Colony PCR with primers ENVR3 (5' CCTCTCGTATAAATACACATTAGGTGATAGATTAACCTTCG 3') and ENVR4 (5' GCAACAGAAACAGACAAATGCCGCAATATG 3') resulted in a 1.2 Kb band or a 0.8 kb if *envR* was disrupted by the Chloramphenicol resistant gene or if *envR* gene was not disrupted, respectively. *ΔenvR* deletion strains were identified and named BW25113 ΔEnvR.

[0234] To generate the lysate, P1 phage lysates were made from BW25113 ΔEnvR as described in Example 3 to allow transfer of the knockouts into the *E. coli* MG1655 monatin production strain.

[0235] For transduction into production hosts, the *envR* deletion was transferred to strain *E. coli* MG1655 containing the plasmid p*aspCproA* ProNdedel as described in Example 3 for *emrB* and *acrAB* knockouts. Strains with the deleted *envR* gene were selected by plating on LB plates containing 25 μg/mL of chloramphenicol and 50 μg/mL of kanamycin. A *ΔenvR* deletion strain was identified and named MG1165 ΔEnvR pApProNdedel.

[0236] To allow the disruptions of other genes in MG1165 ΔEnvR pApProNdedel background, the chloramphenicol resistant marker was excised from the *envR* locus. MG1165 ΔEnvR pApProNdedel was transformed with 15 ng of pCP20 (Datsenko, K.A. and Wanner, B.L., "One-step inactivation of chromosomal genes in *Escherichia coli K-12* using PCR products," *Proceed. Natl. Acad. Sci. USA 97*:6640-6645, (2000)) using the Transformation and Storage Solution protocol (TSS, Epicentre Biotechnologies, Madison, WI) as recommended by the manufacture. After an outgrowth of 30 minutes at 30 °C, the cells were plated on LB plates containing 50μg/mL of ampicillin and 25 μg/mL of kanamycin and incubated overnight at 30 °C. Single colonies were obtain and replated for single colonies on LB plates containing 25 μg/mL of kanamycin and incubated at 42 °C overnight. Single colonies from the previous step, were replica plated on LB containing 25 μg/mL of kanamycin and on plates containing 25 μg/mL of chloramphenicol. Chloramphenicol-sensitive kanamycin-resistant strains were tested for loss of the chloramphenicol marker from the *envR* locus by colony PCR using primers ENV3 and ENVR4 as described above. Amplification of the *envR* locus that had lost the chloramphenicol resistant marker resulted in a 0.3 Kb band in contrast to a 1.2 Kb band produced from the amplification of the *envR* locus containing the chloramphenicol resistant marker. The resulting strain was named MG1165 ΔEnvR pApProNdedel CanS. The *emrB* and or *acrAB* genes can now be disrupted in the same manner as described in Example 3.

[0237] Because the AcrAB and EmrAB transport systems can also transport monatin, the impact of the *envR* deletion, and consequent activation of the AcrEF transport system, on monatin transport, will probably be most

prominent in a host strain that has one, or both, of the AcrAB and EmrAB transport systems deleted.

[0238] Given the structural similarities between indole and monatin, it would be expected that the AcrEF transporter would function for indole-3-pyruvate efflux as well. Consequently, it may be possible that a deletion of the gene that encodes the AcrEF transportermight prevent monatin intermediates from leaking out of the cell; thus allowing more flux into monatin production. At the same time, the AcrEF transporter may function for monatin efflux. Example 25 shows that a transporter that effluxes indole-3- acetic acid also functions for monatin efflux. The decision to inactivate or overexpress the AcrEF transport system will depend on the relative rate of monatin transport to indole-3- pyruvate transport.

EXAMPLE 25

Increase of Monatin Excretion with Overexpression of the *Arabidopsis* Auxin Transporter

[0239] Auxin (primarily indole-3-acetic acid, IAA) is a plant hormone. The directional transport of auxin has been shown to be essential for normal plant growth and development and is mainly mediated by an efflux carrier complex that is characterized by the PIN-FORMED (PIN) family of proteins. Plant orthologs of mammalian multidrug-resistance/P-glycoproteins (MDR/PGPs) also function in auxin efflux. MDR/PGPs are reported to stabilize efflux complexes on the plasma membrane and to function as ATP-dependent auxin transporters. The specificity and directionality of transport is reportedly provided by interacting PIN proteins. Blakeslee J.J., *et al.*, "Auxin transport," *Curr. Opin. Plant Biol. 8*:494-500, (October 2005). Other researchers have suggested an involvement of MDR/PGP-like ABC transporters in transport of the auxin and, AtPGP1 (NP_181228) has been directly implicated in the primary active export of auxin (MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development. Santelia D., *et al.*, *FEBS Lett. 579*:5399-5406, (October 2005); Geisler M., *et al.*, "Cellular efflux of auxin catalyzed by the *Arabidopsis* MDR/PGP transporter AtPGP1," *The Plant Journal 44*:179 (2005)). Because some aspects of

monatin have structural similarities with auxin, the use of auxin transporters to efflux monatin was investigated.

[0240] *Arabidopsis thaliana* mRNA (Cat # M1634310) was obtained from Biochain (Hayward, CA). The mRNA was diluted 10 fold to a final concentration of 50 ng/μL with RNAse free water. cDNA was made from the mRNA using the Reverse Transcription System (Promega, Madison, WI) and Random Primers as described but using 100 ng of mRNA instead of 1 μg of total RNA as template.

[0241] The *AtPGP1* gene was amplified in three parts by PCR using *Pfu Turbo* DNA polymerase (Stratagene, La Jolla, CA) and *Arabidopsis thaliana* cDNA as a template. From base 1 to 1453 of the open reading frame was amplified using primers PGP1 (5' CATATGATGGATAATGACGGTGGTGCTCCTCCTCC 3') and PGP2 (5' CATTTGCGACTCGAGCAGCCTCCTCTATCTC 3'). This introduced an *Nde* I restriction site at the 5' of the open reading frame. An annealing temperature of 63° C and extension time of 2 minutes was used. The PCR fragment was purified by agarose gel electrophoresis and extracted using the QIAquick Gel Extraction Kit (Qiagen Hilden, Germany) as recommended by the manufacture. The purified PCR product was cloned into pCR4.0 Blunt-TOPO (Invitrogen Carlsbad, CA) as recommended the manufacture. The sequence was verified by direct sequencing (Agencourt, Beverly MA). The resulting plasmid was named p*AtPGP1*-5'. From base 1423 to 2829 of the open reading frame was amplified using primers PGP3 (5' GAGATAGAGGAGGCTGCTCGAGTCGCAAATG 3') and PGP4 (5' GAGAGCATAAGATGCATAAAGACAGAACTGAGCTACACC 3'). An annealing temperature of 55 °C and extension time of 2 minutes was used. The PCR fragment was purified, cloned into pCR4.0 Blunt-TOPO and the sequence verified, as described above. The resulting plasmid was named p*AtPGP1*-C. From base 2791 to 3861 of the open reading frame was amplified using primers PGP5 (5' GCGGCCGCCTAAGCATCATCTTCCTTAACCCTAGAACTTGAACCTG AC 3') and primers PGP6 (5' GGTGTAGCTCAGTTCTGTCTTTATGCATCTTATGCTCTC 3'). An annealing temperature of 55° C and extension time of 2 minutes was used.

This introduced a *Not* I restriction site at the end of the open reading frame. The PCR fragment was purified, cloned into pCR4.0 Blunt-TOPO and the sequence verified as described earlier. The resulting plasmid was named pAt-*PGP1*-3'.

[0242] Once the three pieces were individually cloned, the 3' and the center piece were ligated together. The resulting piece was then ligated with the 5' piece to reassemble the full open reading frame and placed in the final plasmid. p*AtPGP1*-C, p*AtPGP1*-3', and pBluescript SK- (Stratagene, La Jolla, CA) were digested with either *Xho* I and *Nsi* I, *Nsi* I and *Not* I, or *Xho* I and *Not* I, respectively. The 1.4 kb (p*AtPGP1*-C), 1.0 Kb (p*AtPGP1*-3') and 3.0 Kb (pBluescript SK-) fragments were purified by agarose gel electrophoresis and extracted as described above. The purified fragments were ligated using Quick Ligation Ligase (New England Biolabs, Ipswich, MA) using the manufacturer's instructions. The resulting plasmid was named p*AtPGP1*-C3'. p*AtPGP1*-C3', *pAtPGP1*-5', and pProNdedel were digested with either *Xho* I and *Not* I, *Nde* I and *Not* I, or *Nde* I and *Not* I, respectively. The 2.4 kb (p*AtPGP1*-C3'), 1.4 Kb (p*AtPGP1*-5') and 2.6 Kb (pProNdedel – we have a procedure that describes the construction of pProNdedel, See Example 15) fragments were purified by agarose gel electrophoresis and extracted as described above. The purified fragments were ligated as described above. The resulting plasmid was named pPro-*AtPGP1* and verified by restriction enzyme analysis.

[0243] Competent *E.coli* Bl21-DE3:: *aspCproA*pET32 were prepared using Transformation and Storage Solution (TSS, Epicentre Biotechnologies). pPro-*AtPGP1* was transformed into competent *E.coli* Bl21-DE3:: *aspCproA*pET32 as recommended by manufacturer and plated in LB plates with 100 μg/mL of ampicillin and 50 μg/mL of kanamycin. The transformants *E.coli* Bl21-DE3:: *aspCproA*pET32 containing a second plasmid pPro-*AtPGP1* were verified by PCR amplification of the 5' and 3' region of *At PGP1*, using primers PGP1 and PGP2 or PGP5 and PGP6, respectively, as described above.

[0244] For inoculum preparation, the *E. coli* strains were grown overnight at 37 °C and 250 rpm in Luria-Bertani ("LB") medium with 100 μg/mL ampicillin and 50 μg/mL kanamycin. For the experimental treatments, Trp-1

+ glucose medium, a minimal medium that has been used for increased production of tryptophan in *E. coli* cells (Zeman, *et al. Folia Microbiol. 35*:200-204, (1990)), was prepared as follows. To 800 mL nanopure water the following reagents were added: 2 g $(NH_4)_2SO_4$ and 13.6 g KH_2PO_4. The pH was adjusted to 7.0, the volume was increased to 948 mL, and the medium was autoclaved. Following sterilization, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$, and 0.5 mg $FeSO_4*7H_2O$ were added to the medium in a 1.8 mL volume followed by addition of 0.2 mL of Neidhardt's micronutrient solution. Neidhardt F.C., *et al.*, "Culture medium for Enterobacteria," *J. Bacteriol. 119*:736-746, (1974). Neidhardt's medium includes (per liter): 0.18 g $(NH_4)_6(MO_7)_{24}$ - $4H_2O$, 1.24 g H_3BO_3, 0.36 g $CoCl_2$ $-6H_2O$, 0.12 g $CuSO_4$ (anhydrous), 0.8 g $MnCl_2 - 4H_2O$, and 0.14 g $ZnSO_4 - 7H_2O$. A 50% glucose solution was prepared separately and sterile-filtered. Forty mL of glucose solution and 10 mL of 1 M 3-Morpholinopropanesulfonic acid ("MOPS") buffer were added to the base medium (950 mL) for a 1 L final volume.

[0245] For treatments, 3.5 v/v % of inoculum was added to 100 mL medium volume in 500 mL baffled shake flasks with 100 µg/mL ampicillin and 50 µg/mL kanamycin. Conditions for the treatments included 250 rpm agitation throughout and 37 °C up to induction, then, 30 °C following induction. At 0.54-0.62 OD_{600nm}, induction of the plasmid genes was initiated. At induction, 1.0 mM IPTG, 0.5% L-arabinose, 0.5 mM pyridoxine hydrochloride, and 0.2 mLs of Balch's vitamins were added. Additions of 10 g/L L-tryptophan, 10 g/L sodium pyruvate, 0.04 mM pyridoxal-5'-phosphate ("PLP") and 0 or 0.2% Tween 20 (polyoxyethylene 20-sorbitan monolaurate) were made 3 hours following induction. Samples for monatin and dry cell weight determination were taken at 24, 30 and 48 hours.

Table 25.1

Increase in Monatin per dry cell weight excreted by *E. coli* due to auxin gene expression

		Monatin / dry cell wt. (mg/g)		
	Tween 20 (%)	24 hours	30 hours	48 hours
E.coli BL21DE3 AP pET32 / no auxin transporter gene on pProNde del	0	1.5	1.6	1.5
E.coli BL21DE3 AP pET32 / no auxin transporter gene on pProNde del	0.2	2.2	2.5	2.2
E.coli BL21DE3 AP pET32 / *Arabidopsis* auxin transporter gene on pProNde del	0	9.6	8.2	6.2
E.coli BL21DE3 AP pET32 / *Arabidopsis* auxin transporter gene on pProNde del	0.2	29.4	26.7*	19.7

n=3 for all treatments except * n=2

[0246] Induction of the *Arabidopsis* auxin transporter gene in strains with the monatin operon on a plasmid resulted in increased monatin per dry cell weight compared to the control treatments with a blank vector (no auxin gene). The 24 hour monatin per dry cell weight result was 9.6 mg/g on average versus 1.5 for the blank vector control without the auxin gene. Additionally, treatment with Tween 20 at 3 hours following induction, resulted in an increased monatin per dry cell weight average of 29.4 mg/g at 24 hours versus 2.2 for the blank vector control. This represents a 13-fold increase in monatin excreted per dry cell weight for the strain expressing the AtPGP1 auxin transporter gene.

[0247] It has been reported that the auxin transporter AtPGP19 (Q9LJX0) has a similar function to AtPGP1 and would also be expected to transport monatin. In addition the literature reports three clusters/clades of p-glycoproteins (PGPs) based on phyogenetic analysis of the Arabidopsis PGPs. At PGP1 is a prototype of Class I, catalyzing auxin transport. In addition to AtPGP1 and AtPGP19, other members of class I PGPs that are expected to play a role in auxin transport include: AtPGP13, AtPGP14, AtPGP10, AtPGP2 from *Arabidopsis* and OsPGP9, OsPGP8, OsPGP7 and Os PGP6 from *Oryza sativa* (rice) ("Geisler, M. and A.S. Murphy, "The ABC of auxin transport: The role of p-glycoproteins in plant development," *FEBS Letters 580*:1094-1102,

(2006)). The PGPs of Class I as mentioned above would all be expected to have some ability for monatin transport.

[0248] In addition a BLAST analysis of the NCBI database using the AtPGP1 protein sequence as a query, indicates that there are a number of homologs such as, but not limited to, Br ABB97035, St AAD10836, Sb AAR10387, Os XP483819, Os CAD59580, ZMPGP1 AAR00316, that could all play a role in monatin transport. The sequence alignment for all of the homologs are shown in FIG. 1.

[0249] Thus, we present strong evidence supporting the role of auxin transporters for monatin efflux.

What is claimed is:

1. A method, comprising: using a genetically-engineered microorganism to secrete monatin.

2. The method of claim 1, wherein said microorganism has been genetically engineered to express or overexpress one or more components of a transporter system capable of secreting monatin.

3. The method of claim 1, wherein said microorganism is genetically-engineered to express one or more components of a transporter system capable of secreting monatin and that is heterologous to said microorganism.

4. The method of claim 1, wherein said micoroorganism is genetically-engineered to overxpress one or more components of a transporter system capable of secreting monatin and that is native to said microorganism.

5. The method of claim 1, wherein said microorganism is genetically engineered to synthesize monatin.

6. The method of claim 1, wherein the microorganism contains one or more transporter systems chosen from the AcrAB transporter system, homologs of the AcrAB transporter system, the EmrAB transporter system, and homologs of the EmrAB transporter system.

7. A method, comprising: producing monatin in a microorganism; inducing said microorganism to express or overexpress a transporter system that is capable of secreting said monatin or components of a transporter system that are capable of secreting said monatin; and, secreting said monatin from said microorganism.

8. The method of claim 7, wherein said transporter system is the AcrAB transporter system and the inducing step comprises providing sodium decanoate to the culture medium.

9. The method of claim 7, wherein said transporter system is the EmrAB transporter system and the inducing step comprises providing carbonyl cyanide 3-chlorophenylhydrazone to the culture medium.

10. The method of claim 7, wherein the secretion of monatin is an increased secretion of monatin as compared to an appropriate control.

11. A method, comprising: producing monatin *in vivo* using a microorganism genetically engineered to express or overexpress one or more proteins chosen from TolC, RobA, RamA, MarA, BaeR, and homologs thereof.

12. A method, comprising: producing monatin in a glutamate auxotroph.

13. The method of claim 12, wherein said microorganism is genetically engineered to produce monatin, genetically engineered to express or overexpress one or more types of transporter systems capable of secreting monatin, or both.

14. A method, comprising: producing monatin in a microorganism capable of translocating glutamate or solute similar to glutamate in exchange for malate.

15. The method of claim 7, wherein the inducing step comprises providing salicylate to the culture medium.

16. A method, comprising: producing monatin in a microorganism that produces or is genetically engineered to produce or overproduce auxin transporters, homologs of auxin transporters or combinations thereof.

17. The method of claim 16, wherein said homologs of said auxin transporters comprises an amino acid sequence having the consensus sequence shown in FIG. 1 when aligned as shown in FIG.1 with AtPGP1, BrABB97035, StAAD10836, ZmPGP1_AAR00316, SbAAR10387, OsXP_483819, OS_CAD59580 and AtPGP19.

18. The method of claim 16, wherein said homologs of said auxin transporters comprise one or more amino acid sequences chosen from PXGKTXAXVGXSGSGKSTVVSLXERFYXPXXGXXXLDG, LXLXXLRXQIGLVXQEPXLFATXIXENXLG, and QVGERGXQLSGGQKQRIAIARAMLXXPXILLLDEATSALD, wherein X is an amino acid at the indicated position of any one of AtPGP1, BrABB97035, StAAD10836, ZmPGP1_AAR00316, SbAAR10387, OsXP_483819, OS_CAD59580, and AtPGP19 when aligned with said amino acid sequence, as shown in FIG. 1B.

19. The method of claim 16, wherein said homologs of said auxin transporters comprise one or more amino acid sequences chosen from LPXGYXTXVGERGVQLSGGQXQRIAIARA and LLDEATSALDAESEXXXQEAL, wherein X is an amino acid at the indicated position of any one of AtPGP1, BrABB97035, StAAD10836, ZmPGP1_AAR00316, SbAAR10387, OsXP_483819, OS_CAD59580, and AtPGP19 when aligned with said amino acid sequence, as shown in FIG. 1D.

20. A method, comprising: producing monatin in a microorganism lacking a *cysH* gene.

21. The method of claim 20, wherein said microorganism is genetically engineered to lack the *cysH* gene .

22. A method, comprising: producing monatin using a microorganism genetically engineered to be deficient in, or depressed in, levels of mycolic acids in its cell envelope as compared to said microorganism before the genetic engineering.

23. The method of claim 1, wherein said microorganism is a member of the *Pantoea* family.

24. The method of claim 1, wherein said microorganism is a member of the *Corynebacterium* family .

25. The method of claim 7, wherein said microorganism is exposed to a compound that increases monatin production, monatin efflux, or both monatin production and monatin efflux as compared with an appropriate control.

26. The method of claim 25, wherein the compound is chosen from ampicillin, ethambutol, pyruvate, Tween, and combinations thereof.

27. The method of claim 1, wherein said genetically-engineered organism lacks one or more transporters chosen from YhcP (AaeB), YccS, YjcQ and YhfK.

28. A method, comprising identifying a monatin transporter by verifying transporter efficacy for secreting monatin.

29. A genetically engineered microorganism capable of secreting monatin.

30. The microorganism of claim 29, wherein said microorganism is genetically engineered to produce or overproduce monatin.

31. The microorganism of claim 29, wherein said microorganism is genetically engineered to express or overexpress one or more transporter systems capable of secreting monatin.

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AtPGP1              ----------------------------------------------------MDNDGGA
BrABB97035          --------------MQGLELLPE--------PSSNSNSNSRNPETELQEHPPEMGNGGGT
StAAD10836          --------------MQGVELVVS--------EDKNSN----TPTTTTTTNSHQFQETRME
ZmPGP1_AAR00316     MSSSDPEEIRARVVVLGSPHAD--GGDEWARPELEAFHLPSPAHQPPGFLAG---QPEAA
SbAAR10387          MSTNDPDEIRARVVVLGAPHADDDAGDEWARPELEAFHLPSPAHQPPGFHLAAGHQPEAA
OsXP_483819         ----MEEEIKGRVVVLGADAAAD--------PELEAFHLPSADQPPHSHLLHHHHSPQSH
OS_CAD59580         ----MPPPTRSSRPSISP-PPTS--------PRTRTSSTTTILHNPILNLMHQQQQRHRH
AtPGP19             ----------------------------------------------------MSETNTT
Consensus/100%      ....................................................

AtPGP1              PPPPPTLVVEEPKK------------------------------------------------
BrABB97035          PPPPPPATVEEPKK------------------------------------------------
StAAD10836          VKKEEGGDVEKPS-------------------------------------------------
ZmPGP1_AAR00316     EQPTLPAPAGRSSS(5)TTSAGGGAAPPPPSSPPPPPASLETEQPPNARPAS---AGAND
SbAAR10387          AEQPTTLPAARRTS---DTSTAAGAAPPSPS-PPPPPAPLEMDQPPNAKPAS(5)AGAND
OsXP_483819         PQPDAPAAAAPPPP-------APLTPPPPKSPPPPPHIQTTDLPPP--------------
OS_CAD59580         LLPLLLLLLSRRRR-------LPTSKPP-------------TFHRP--------------
AtPGP19             DAKTVPAEAEKKKE------------------------------------------------
Consensus/100%      ....................................................

AtPGP1              --AEIRGVAFKELFRFADGLDYVLMGIGSVGAFVHGCSLPLFLRFFADLVNSFGSNSNNV
BrABB97035          --AEIRGVAFKELFRFADGLDYVLMTIGSVGAFVHGCSLPLFLRFFADLVNSFGSNANNV
StAAD10836          --SPPPAVGFGELFRFADGLDCVLMIIGSLGAFVHGCSLPLFLRFFADLVNSFGSYANDV
ZmPGP1_AAR00316     SKKPTPPAALRDLFRFADGLDCALMLIGTLGALVHGCSLPVFLRFFADLVDSFGSHADDP
SbAAR10387          NKKPTPPAALRDLFRFADGLDCALMLVGTLGALVHGCSLPVFLRFFADLVDSFGSHANDP
OsXP_483819         --KPLPPAPLRQLFSFADGLDYVLMTLGTLGALVHGCSLPVFLRFFADLVDSFGSHAAHP
OS_CAD59580         --RPLPPAPFRQLFSFGDGLDYVLMTLGTLGALVHGCSLTVFLRFFADLVDSFGSHAAHP
AtPGP19             -----QSLPFFKLFSFADKFDYLLMFVGSLGAIVHGSSMPVFFLLFGQMVNGFGKNQMDL
Consensus/100%      ............LF.F.D..D..LM..G..GA.VHG.S...F...F...V..FG......

AtPGP1              EKMMEEVLKYALYFLVVGAAIWASSWAEISCWMWSGERQTTKMRIKYLEAALNQDIQFFD
BrABB97035          DKMMQEVLKYALYFLVVGAAIWASSWAEISCWMWTGERQTTKMRIKYLEAALNQDIQFFD
StAAD10836          DKMTQEVLKYAFYFLVVGAAIWASSWAEISCWMWTGERQTTKMRIKYLEAALNQDIQYFD
ZmPGP1_AAR00316     DTMVRLVVKYAFYFLVVGAAIWASSWAEISCWMWTGERQSTRMRIRYLDAALRQDVSFFD
SbAAR10387          DTMVRLVVKYAFYFLVVGAAIWASSWAEISCWMWTGERQSTRMRIRYLDAALRQDVSFFD
OsXP_483819         DTMLRLVVKYAFYFLVVGAAIWASSWAEISCWMWTGERQSTRMRIRYLHAALHQDVSFFD
OS_CAD59580         DTMLRLVVKYAFYFLVVGAAIWASSWAEISCWMWTGERQSTRMRIRYLHAALHQDVSFFD
AtPGP19             HQMVHEVSRYSLYFVYLGLVVCFSSYAEIACWMYSGERQVAALRKKYLEAVLKQDVGFFD
Consensus/100%      ..M...V..Y..YF...G.....SS.AEI.CWM..GERQ....R..YL.A.L.QD...FD

AtPGP1              TEVRTSDVVFAINTDAVMVQDAISEKLGNFIHYMATFVSGFIVGFTAVWQLALVTLAVVP
BrABB97035          TEVRTSDVVSAINTDAVMVQDAISEKLGNFIHYMA----------------LVTIAVVP
StAAD10836          TEVRTSDVVSAINTDAVVVQDAISEKLGNFIHYMATFLSGFVVGFTAVWQLALVTLAVVP
ZmPGP1_AAR00316     TDVRASDVIYAINADAVVVQDAISQKLGNLIHYMATFVAGFVVGFTAAWQLALVTLAVVP
SbAAR10387          TDVRTSDVIYAINADAVVVQDAISEKLGNLIHYMATFVAGFVVGFTAAWQLALVTLAVVP
OsXP_483819         TDVRTSDVIHAINADAVVVQDAISEKLGNLIHYLATFVSGFVVGFTAAWQLALVTLAVVP
OS_CAD59580         TDVRTSDVIHAINADAVVVQDAISEKLGNLIHYLATFVSGFVVGFTAAWQLALVTLAVVP
AtPGP19             TDARTGDIVFSVSTDTLLVQDAISEKVGNFIHYLSTFLAGLVVGFVSAWKLALLSVAVIP
Consensus/100%      T..R..D.......D...VQDAIS.K.GN.IHY..................L...AV.P

AtPGP1              LIAVIGGIHTTTLSKLSNKSQESLSQAGNIVEQTVVQIRVVMAFVGESRASQAYSSALKI
BrABB97035          LIAVIGGIHTTTLSKLSNKSQESLSQAGNIVEQTVVQIRVVMAFVGESRASQAYSSALKT
StAAD10836          LIAVIGAIYTVTSAKLSSQSQEALSKAGNIVEQTVVQIRTVLVFVGEAKALQAYTAALRV
ZmPGP1_AAR00316     LIAVIGGLSAAALAKLSSRSQDALSGASGIAEQALAQIRIVQAFVGEEREMRAYSAALAV
SbAAR10387          LIAVIGGLSAAALAKLSSRSQDALSGASGIAEQALAQIRIVQAFVGEEREMRAYSAALAV
OsXP_483819         LIAVIGGLSAAALAKLSSRSQDALSDASGIAEQALAQIRIVQSFVGEERVMRAYSAALAV
OS_CAD59580         LIAVIGGLSAAALAKLSSRSQDALSDASGIAEQALAQIRIVQSFVGEERVMRAYSAALAV
AtPGP19             GIAFAGGLYAYTLTGITSKSRESYANAGVIAEQAIAQVRTVYSYVGESKALNAYSDAIQY
Consensus/100%      .IA..G............S......A..I.EQ...Q.R.V...VGE.....AY..A...
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FIG. 1A

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AtPGP1              AQKLGYKTGLAKGMGLGATYFVVFCCYALLLWYGGYLVRHHLTNGGLAIATMFAVMIGGL
BrABB97035          AQKLGYKTGFAKGMGLGATYFVVFCCYALLLWYGGYLVRHHLTNGGLAIATMFAVMIGGL
StAAD10836          SQKIGYKSGFSKGLGLGATYFTVFCCYALLLWYGGYLVRHHFTNGGLAIATMFAVMIGGL
ZmPGP1_AAR00316     AQRIGYRSGFAKGLGLGGTYFTVFCCYGLLLWYGGHLVRAQHTNGGLAIATMFSVMIGGL
SbAAR10387          AQKIGYRSGFAKGLGLGGTYFTVFCCYGLLLWYGGHLVRGHHTNGGLAIATMFSVMIGGL
OsXP_483819         AQRIGYRSGFAKGIGLGGTYFTVFCCYALLLWYGGHLVRRAHTNGGLAIATMFSVMIGGL
OS_CAD59580         AQRIGYRSGFAKGIGLGGTYFTVFCCYALLLWYGGHLVRRAHTNGGLAIATMFSVMIGGL
AtPGP19             TLKLGYKAGMAKGLGLGCTYGIACMSWALVFWYAGVFIRNGQTDGGKAFTAIFSAIVGGM
Consensus/100%      ....GY..G..KG.GLG.TY........L..WY.G...R...T.GG.A....F....GG.

AtPGP1              ALGQSAPSMAAFAKAKVAAAKIFRIIDHKPTIERNSES---GVELDSVTGLVELKNVDFS
BrABB97035          GLGQSVPSMAAFAKAKVAAAKIFRIIDHKPTIERNSES---GVELESVTGLVELKNVDFS
StAAD10836          ALGQSAPSMTAFAKARVAAAKIFRIIDHKPSVDRNAKT---GLELDTVSGQLELKNVEFS
ZmPGP1_AAR00316     PR-QSAPSMAAFAKARVAAAKIFRIIDHRPGISSRDG-----AEPESVTGRVEMRGVDFA
SbAAR10387          ALGQSAPSMAAFAKARVAAAKIFRIIDHRPGISSRDGEDGGGVELESVTGRVEMRGVDFA
OsXP_483819         ALGQSAPSMAAFAKARVAAAKIFRMMEHKPSMEREGG-----VELEAVTGRVELRDVEFS
OS_CAD59580         ALGQSAPSMAAFAKARVAAAKIFRMMEHKPSMEREGG-----VELEAVTGRVELRDVEFS
AtPGP19             SLGQSFSNLGAFSKGKAAGYKLMEIINQRPTIIQDPLDG---KCLDQVHGNIEFKDVTFS
Consensus/100%      ...QS.....AF.K...A..K........P.................V.G..E...V.F.

AtPGP1              YPSRPDVKILNNFCLSVPAGKTIALVGSSGSGKSTVVSLIERFYDPNSGQVLLDGQDLKT
BrABB97035          YPSRPDVKILNDFTLSVPAGKTIALVGSSGSGKSTVVSLIERFYDPTSGQVLLDGHDLKT
StAAD10836          YPSRPEIKILNNFNLVVPAGKTIALVGSSGSGKSTVVSLIERFYDPTSGQLMLDGNDIKT
ZmPGP1_AAR00316     YPSRPDVPILRGFSLSVPAGKTIALVGSSGSGKSTVVSLIERFYDPSAGQILLDGHDLRS
SbAAR10387          YPSRPDVPILRGFSLSVPAGKTIALVGSSGSGKSTVVSLLERFYDPSAGQILLDGHDLKS
OsXP_483819         YPSRPDVGILRGLSLSVPAGKTIALVGSSGSGKSTVVSLIERFYEPNAGTILLDGHDLRD
OS_CAD59580         YPSRPDVGILRGLSLSVPAGKTIALVGSSGSGKSTVVSLIERFYEPNAGTILLDGHDLRD
AtPGP19             YPSRPDVMIFRNFNIFFPSGKTVAVVGGSGSGKSTVVSLIERFYDPNSGQILLDGVEIKT
Consensus/100%      YPSRP...I........P.GKT.A.VG.SGSGKSTVVSL.ERFY.P..G...LDG.....

AtPGP1              LKLRWLRQQIGLVSQEPALFATSIKENILLGRPD--ADQVEIEEAARVANAHSFIIKLPD
BrABB97035          LKLKWLRQQIGLVSQEPALFATSIKENILLGRPD--ADQVEVEEAARVANAHSFIIKLPD
StAAD10836          LKLKWLRQQIGLVSQEPALFATSIKENILLGRPD--ATQIEIEEAARVANAHSFVIKLPD
ZmPGP1_AAR00316     LELRWLRRQIGLVSQEPALFATSIRENLLLGRDSQSATLAEMEEAARVANAHSFIIKLPD
SbAAR10387          LKLRWLRQQIGLVSQEPTLFATSIKENLLLGRDSQSATQAEMEEAARVANAHSFIVKLPD
OsXP_483819         LNLRWLRRQIGLVSQEPALFATTIRENLLLGRDG--ATQEELEEAARVANAHSFIVKLPD
OS_CAD59580         LNLRWLRRQIGLVSQEPALFATTIRENLLLGRDG--ATQEELEEAARVANAHSFIVKLPD
AtPGP19             LQLKFLREQIGLVNQEPALFATTILENILYGKPD--ATMVEVEAAASAANAHSFITLLPK
Consensus/100%      L.L..LR.QIGLV.QEP.LFAT.I.EN.L.G.....A...E.E.AA..ANAHSF...LP.

AtPGP1              GFDT----QVGERGLQLSGGQKQRIAIARAMLKNPAILLLDEATSALDSESEKLVQEALD
BrABB97035          GFDT----QVGERGLQLSGGQKQRIAIARAMLKNPAILLLDEATSALDSESEKLVQEALD
StAAD10836          GFDT----QVGERGLQLSGGQKQRIAIARAMLKNPAILLLDEATSALDSESEKLVQEALD
ZmPGP1_AAR00316     GYDT----QVGERGLQLSGGQKQRIAIARAMLKNPAILLLDEATSALDSESEKLVQEALD
SbAAR10387          GYDT----QVGERGLQLSGGQKQRIAIARAMLKNPAILLLDEATSALDSESEKLVQEALD
OsXP_483819         AYNT----QVGERGLQLSGGQKQRIAIARAMLRNPAILLLDEATSALDSESEKLVQEALD
OS_CAD59580         AYNT(19)QVGERGLQLSGGQKQRIAIARAMLRNPAILLLDEATSALDSESEKLVQEALD
AtPGP19             GYDT----QVGERGVQLSGGQKQRIAIARAMLKDPKILLLDEATSALDASSESIVQEALD
Consensus/100%      ...T....QVGERG.QLSGGQKQRIAIARAML..P.ILLLDEATSALD..SE..VQEALD

AtPGP1              RFMIGRTTL-IIAHRLSTIRKADLVAVLQQGSVSEIGTHDELFSKGENGVYAKLIKMQEA
BrABB97035          RFMIGRTTL-IIAHRLSTIRKADLVAVLQQGSVSEIGTHDELFAKGENGIYSKLIKMQEA
StAAD10836          RFMIGRTTL-VIAHRLSTIRKADLVAVLQQGSVSEIGSHDELMSKGENGMYAKLIKMQEA
ZmPGP1_AAR00316     RFMMGRTTLGDRATGCPPSAKADVVAVLQGGAVSEMSAHDELMAKGENGTYAKLIRMQEQ
SbAAR10387          RFMIGRTTL-VIAHRMSTIRKADVVAVLQGGPVSEMGAHDELMAKGENGTYAKFIRMQEQ
OsXP_483819         RFMIGRTTL-VIAHRLSTIRKADLVAVLQGGAISEVGTHDELMARGD-GTYARLIRMQEQ
OS_CAD59580         RFMIGRTTL-VIAHRLSTIRKADLVAVLQGGAISEVGTHDELMARGD-GTYARLIRMQEQ
AtPGP19             RVMVGRTTV-VVAHRLCTIRNVDSIAVIQQGQVVETGTHEELIAKSG--AYASLIRFQEM
Consensus/100%      R.M.GRTT....A.........D..AV.Q.G...E...H.EL........Y...I..QE.
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FIG. 1B

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AtPGP1            AHETAMSNARKSSARPSSARNSVSSPIMTRNSSYGRSPYSRRLSDFS-TSDFSLSIDASS
BrABB97035        AHETAMNNARKSSARPSSARNSVSSPIIARNSSYGRSPYSRRLSDFS-TTDFSLSVEASS
StAAD10836        AHETALSNARKSSARPSSARNSVSSPIITRNSSYGRSPYSRRLSDFS-TSDFSLSLDAA-
ZmPGP1_AAR00316   AHEAALVNARRSSARPSSARNSVSSPIMTRNSSYGRSPYSRRLSDFS-TSDFTLSIHDPH
SbAAR10387        AHEAAFVNARRSSARPSSARNSVSSPIMTRNSSYGRSPYSRRLSDFS-TSDFTLSIHDPH
OsXP_483819       AHEAALVAARRSSARPSSARNSVSSPIITRNSSYGRSPYSRRLSDADFITGLGLGVDSKQ
OS_CAD59580       AHEAALVAARRSSARPSSARNSVSSPIITRNSSYGRSPYSRRLSDADFITGLGLGVDSKQ
AtPGP19           VGTRDFSNPSTRRTRSTRLSHSLSTKSLSLRSGSLRNLSYSYSTGADGRIEMISNAETDR
Consensus/100%    .............R......S.S.......S...R.....................

AtPGP1            YPNYR--NEKLAFKDQANSFWRLAKMNSPEWKYALLGSVGSVICGSLSAFFAYVLSAVLS
BrABB97035        YPNYR--HDKLPFKDQANSFWRLAKMNSPEWKYALVGSVGSVICGSLSAFFAYVLSAVLS
StAAD10836        YSNYR--NEKLAFKDQASSFGRLAKMNSPEWTYALIGSIGSVICGSLSAFFAYVLSAVLS
ZmPGP1_AAR00316   HHHRTMADKQLAFRAGASSFLRLARMNSPEWAYALAGSIGSMVCGSFSAIFAYILSAVLS
SbAAR10387        HHHRTMADKQLAFRAGASSFLRLARMNSPEWAYALVGSLGSMVCGSFSAIFAYILSAVLS
OsXP_483819       QQQ------QHYFRVQASSFWRLAKMNSPEWGYALVASLGSMVCGSFSAIFAYVLSAVLS
OS_CAD59580       QQQ------QHYFRVQASSFWRLAKMNSPEWGYALVASLGSMVCGSFSAIFAYVLSAVLS
AtPGP19           KTR----------APENYFYRLLKLNSPEWPYSIMGAVGSILSGFIGPTFAIVMSNMIE
Consensus/100%    ..................F.RL...NSPEW.Y......GS...G.....FA...S....

AtPGP1            VYYNPDHEYMIKQIDKYCYLLIGLSSAALVFNTLQHSFWDIVGENLTKRVREKMLSAVLK
BrABB97035        IYYNPDHNYMIKQIDKYCYLLIGLSSAALIFNTLQHSFWDIVGENLTKRVREKMLTAVLK
StAAD10836        VYYNPDHAYMSEQIAKYCYLLIGVSSAALIFNTLQHYYWDVVGENLTKRVREKMLAAVLK
ZmPGP1_AAR00316   VYYAPDPRYMKREIAKYCYLLIGMSSAALLFNTVQHVFWDTVGENLTKRVREKMFAAVFR
SbAAR10387        VYYAPDPRYMKREIAKYCYLLIGMSSAALLFNTVQHVFWDTVGENLTKRVREKMFAAVLR
OsXP_483819       VYYAPDAAYMDRQIAKYCYLLIGMSSAALLFNTVQHLFWDTVGENLTKRVRERMLAAVLR
OS_CAD59580       VYYAPDAAYMDRQIAKYCYLLIGMSSAALLFNTVQHLFWDTVGENLTKRVRERMLAAVLR
AtPGP19           VFYYTDYDSMERKTKEYVFIYIGAGLYAVGAYLIQHYFFSIMGENLTTRVRRMMLSAILR
Consensus/100%    ..Y..D...M......Y....IG....A......QH......GENLT.RVR..M..A...

AtPGP1            NEMAWFDQEENESARIAARLALDANNVRSAIGDRISVIVQNTALMLVACTAGFVLQWRLA
BrABB97035        NEMAWFDQEENESARISARLALDANNVRSAIGDRISVIVQNTALMLVACTAGFVLQWRLA
StAAD10836        MEMAWFDQEENDSSRIAARLSLDANNVRSAIGDRISVIMQNSALMLVACTAGFVLQWRLA
ZmPGP1_AAR00316   NEIAWFDADENASARVTARLALDAQNVRSAIGDRISVIVQNSALMLVACTAGFVLQWRLA
SbAAR10387        NEIAWFDADENASARVAARLALDAQNVRSAIGDRISVIVQNSALMLVACTAGFVLQWRLA
OsXP_483819       NEIAWFDMEDNSSARIAARLALDAQNVRSAIGDRISIIVQNSALMLVACTAGFVLQWRLA
OS_CAD59580       NEIAWFDMEDNSSARIAARLALDAQNVRSAIGDRISIIVQNSALMLVACTAGFVLQWRLA
AtPGP19           NEVGWFDEDEHNSSLIAARLATDAADVKSAIAERISVILQNMTSLLTSFIVAFIVEWRVS
Consensus/100%    .E..WFD.....S....ARL..DA..V.SAI..RIS.I.QN....L......F...WR..

AtPGP1            LVLVAVFPVVVAATVLQKMFMTGFSGDLEAAHAKGTQLAGEAIANVRTVAAFNSEAKIVR
BrABB97035        LVLVAVFPVVVAATVLQKMFMTGFSGDLEAAHAKGTQLAGEAIANVRTVAAFNSEAKIVR
StAAD10836        LVLIGVFPVVVAATVLQKMFMKGFSGDLEAAHAKATQLAGEAVANVRTVAAFNSETKIVN
ZmPGP1_AAR00316   LVLLAVFPLVVGATVLQKMFMKGFSGDLEAAHARATQIAGEAVANLRTVAAFNAERKITG
SbAAR10387        LVLLAVFPLVVAATVLQKMFMKGFSGDLEAAHARATQIAGEAVANLRTVAAFNAERKITG
OsXP_483819       LVLLAVFPLVVAATVLQKMFLKGFSGDLERAHARATQIAGEAVANVRTVAAFGSEAKIVG
OS_CAD59580       LVLLAVFPLVVAATVLQKMFLKGFSGDLERAHARATQIAGEAVANVRTVAAFGSEAKIVG
AtPGP19           LLILGTFPLLVLANFAQQLSLKGFAGDTAKAHAKTSMIAGEGVSNIRTVAAFNAQSKILS
Consensus/100%    L.....FP..V.A...Q.....GF.GD...AHA.....AGE...N.RTVAAF....KI..

AtPGP1            LYTANLEPPLKRCFWKGQIAGSGYGVAQFCLYASYALGLWYASWLVKHGISDFSKTIRVF
BrABB97035        LYTANLEPPLKRCFWKGQIAGSGYGVAQFCLYASYALGLWYASWLVKHGISDFSKTIRVF
StAAD10836        LFDSSLQTPLRRCFWKGQIAGSGYGIAQFLLYSSYALGLWYASWLVKHGISDFSKTIRVF
ZmPGP1_AAR00316   LFEANLRGPLRRCFWKGQIAGSGYGVAQFLLYASYALGLWYAAWLVKHGVSDFSRTIRVF
SbAAR10387        LFEANLRGPLRRCFWKGQIAGSGYGVAQFLLYASYALGLWYAAWLVKHGVSDFSRTIRVF
OsXP_483819       LFEANLAGPLRRCFWKGQIAGSGYGVAQFLLYASYALGLWYAAWLVKHGVSDFSKTIRVF
OS_CAD59580       LFEANLAGPLRRCFWKGQIAGSGYGVAQFLLYASYALGLWYAAWLVKHGVSDFSKTIRVF
AtPGP19           LFCHELRVPQKRSLYRSQTSGFLFGLSQLALYGSEALILWYGAHLVSKGVSTFSKVIKVF
Consensus/100%    L....L..P..R.....Q..G...G..Q..LY.S.AL.LWY...LV..G.S.FS..I.VF
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FIG. 1C

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AtPGP1              MVLMVSANGAAETLTLAPDFIKGGQAMRSVFELLDRKTEIEPDDPDTTPVPDRLRGEVEL
BrABB97035          MVLMVSANGAAETLTLAPDFIKGGQAMRSVFELLDRKTEIEPDDLDTTPVPDRLRGEVEL
StAAD10836          MVLMVSANGAAETLTLAPDFIKGGRAMRSVFELLDRKTEVEPDDPDATAVPDRLRGEVEF
ZmPGP1_AAR00316     MVLMVSANGAAETLTLAPDFIKGGRAMRSVFETIDRKTEVEPHDVDAAPVPDGPGAKVEL
SbAAR10387          MVLMVSANGAAETLTLAPDFVKGGRAMRSVFETIDRKTEVEPDDVDAAPVPERPKGEVEL
OsXP_483819         MVLMVSANGAAETLTLAPDFVKGGRAMQAVFEAMDRRTEIEPDDVDAAAVPERPRGEVEL
OS_CAD59580         MVLMVSANGAAETLTLAPDFVKGGRAMQAVFEAMDRRTEIEPDDVDAAAVPERPRGEVEL
AtPGP19             VVLVITANSVAETVSLAPEIIRGGEAVGSVFSVLDRQTRIDPDDADADPV-ETIRGDIEF
Consensus/100%      .VL...AN..AET..LAP....GG.A...VF...DR.T...P.D.D...V........E.

AtPGP1              KHIDFSYPSRPDIQIFRDLSLRARAGKTLALVGPSGCGKSSVISLIQRFYEPSSGRVMID
BrABB97035          KHIDFSYPSRPDIQVFRDLSLRARAGKTLALVGPSGCGKSSVISLIQRFYEPSSGRVLID
StAAD10836          KHVDFSYPTRPDVSIFRDLNLRARAGKTLALVGPSGCGKSSVISLIQRFYEPSSGRVLID
ZmPGP1_AAR00316     KHVDFLYPSRPDIQVFRDLSLRARAGKTLALVGPSGSGKSSVLALVQRFYKPTSGRVLLD
SbAAR10387          KHVDFSYPSRPDIQVFRDLSLRARAGKTLALVGPSGCGKSSVLALVQRFYEPTSGRVLLD
OsXP_483819         KHVDFAYPSRPEVQVFRDLSLRARAGRTLALVGASGCGKSSVLALVQRFYEPNSGRVLLD
OS_CAD59580         KHVDFAYPSRPEVQVFRDLSLRARAGRTLALVGASGCGKSSVLALVQRFYEPNSGRVLLD
AtPGP19             RHVDFAYPSRPDVMVFRDFNLRIRAGHSQALVGASGSGKSSVIAMIERFYDPLAGKVMID
Consensus/100%      .H.DF.YP.RP....FRD..LR.RAG...ALVG.SG.GKSSV.....RFY.P..G.V..D

AtPGP1              GKDIRKYNLKAIRKHIAIVPQEPCLFGTTIYENIAYGHECATEAEIIQAATLASAHKFIS
BrABB97035          GKDIRKYNLKAIRKHIAIVPQEPCLFGTTIYENIAYGHECATEAEIIQAATLASAHKFIS
StAAD10836          GKDIRKYNLKSLRRHIAVVPQEPCLFATTIYENIAYGHESATEAEITEAATLANAHKFIS
ZmPGP1_AAR00316     GKDVRKYNLRALRRVVAVVPQEPFLFAASIHENIAYGREGATEAEVVEAAAQANAHRFIA
SbAAR10387          GKDVRKYNLRALRRVVAVAPQEPFLFAASIHDNIAYGREGATEAEVVEAATQANAHRFIA
OsXP_483819         GRDLRKFNLRSLRRAMALVPQEPFLFAATIHDNIAYGREGATEAEVVEAATAANAHKFIS
OS_CAD59580         GRDLRKFNLRSLRRAMALVPQEPFLFAATIHDNIAYGREGATEAEVVEAATAANAHKFIS
AtPGP19             GKDIRRLNLKSLRLKIGLVQQEPALFAATIFDNIAYGKDGATESEVIDAARAANAHGFIS
Consensus/100%      G.D.R..NL...R.......QEP.LF...I..NIAYG...ATE.E...AA..A.AH.FI.

AtPGP1              ALPEGYKTYVGERGVQLSGGQKQRIAIARALVRKAEIMLLDEATSALDAESERSVQEALD
BrABB97035          ALPDGYKTYVGERGVQLSGGQKQRIAIARALVRKAEIMLLDEATSALDAESERSVQEALD
StAAD10836          ALPDGYKTFVGERGVQLSGGQKQRIAIARAFLRKAELMLLDEATSALDAESERCVQEALD
ZmPGP1_AAR00316     ALPEGYRTQVGERGVQLSGGQRQRIAIARALVKQAAIVLLDEATSALDAESERCVQEALE
SbAAR10387          ALPEGYGTQVGERGVQLSGGQRQRIAIARALVKQAAIVLLDEATSALDAESERCVQEALE
OsXP_483819         ALPEGYGTLVGERGVQLSGGQRQRIAIARALVKQAPILLLDEATSALDAESERCVQEALE
OS_CAD59580         ALPEGYGTLVGERGVQLSGGQRQRIAIARALVKQAPILLLDEATSALDAESERSVQEALA
AtPGP19             GLPEGYKTPVGERGVQLSGGQKQRIAIARAVLKNPTVLLLDEATSALDAESECVLQEALE
Consensus/100%      .LP.GY.T.VGERGVQLSGGQ.QRIAIARA........LLDEATSALDAESE...QEAL.

AtPGP1              Q-ACSGRTSIVVAHRLSTIRNAHVIAVIDDGKVAEQGSHSHLLKNHPDGIYARMIQLQRF
BrABB97035          Q-ACSGRTSIVVAHRLSTIRNAHVIAVIDDGKVVEQGSHSHLLKNYPDGIYARMIQLQRF
StAAD10836          R-ACAGKTTIVVAHRLSTIRNAHVIAVIDDGKVAEQGSHSHLLKNYSDGIYARMIQLQRF
ZmPGP1_AAR00316     R-AGSGRTTIVVAHRLATVRGAHTIAVIDDGKVAEQGSHSHLLKHHPDGCYARMLQLAAA
SbAAR10387          R-AGSGRTTIVVAHRLATVRGAHTIAVIDDGKVAEQGSHSHLLKHHPDGCYARMLQLQRL
OsXP_483819         SSSGSGRTTIVVAHRLATVRNAHTIAVIDDGKVAEQGSHSHLLNHHPDGCYARMLQLQRL
OS_CAD59580         SSSGSGRTTIVVAHRLATVRNAHTIAVIDDGKVAEQGSHSHLLNHHPDGCYARMLQLQRL
AtPGP19             R-LMRGRTTVVVAHRLSTIRGVDCIGVIQDGRIVEQGSHSELVS-RPEGAYSRLLQLQTH
Consensus/100%      .....G.T..VVAHRL.T.R....I.VI.DG...EQGSHS.L......G.Y.R..QL...

AtPGP1              THTQVIGMTSG--SSSRVKEDDA----------
BrABB97035          THTQVIGMTSG--SSSRVKEDDA----------
StAAD10836          THGEAVNMATGSTSSSRPKEDQD----------
ZmPGP1_AAR00316     DGRGGR-ARAVVLVQRGRVGRNGWMDGFGSSRD-
SbAAR10387          TGGCRA-RAAAVVVQRGR--RVGWMDGSWMSLVP
OsXP_483819         SHSHVAPGPSSSTTTHGT---------------
OS_CAD59580         SHSHVAPGPSSSTTTHGT---------------
AtPGP19             RI-------------------------------
Consensus/100%      ................................
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FIG. 1D