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(54) Title: SEQUENCES FOR PRODUCTION OF 2,4-DIACETYLPHLOROGLUCINOL AND METHODS (57) Abstract <p>DNA sequences which function specifically in the synthesis of 2,4-diacetylphloroglucinol (Phl) are described. The sequences include <i>phl</i> genes which encode <i>phl</i> gene proteins and coding and regulatory sequences for production of Phl as well as sequences containing <i>phl</i> genes, which sequences have the capability of conferring or enhancing Phl biosynthetic capability in bacterial strains. The transformed strains are useful as biocontrol agents against fungal pathogens.</p>		

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SEQUENCES FOR PRODUCTION OF 2,4-DIACETYLPHLOROGLUCINOL AND
METHODS

FIELD OF THE INVENTION

The present invention relates to materials and methods for improved control of plant pathogens. More particularly, the present invention relates to the identification, isolation, cloning, and sequencing of genetic elements which confer or enhance the ability of an organism to produce the antibiotic 2,4-diacetylphloroglucinol, and uses thereof, particularly the use to transform an organism to introduce or enhance 2,4-diacetylphloroglucinol biosynthetic capability.

BACKGROUND OF THE INVENTION

Rhizobacteria are plant-associated bacteria, derived from many genera, that have the ability to colonize roots aggressively (Schroth and Hancock, *Ann. Rev. Microbiol.* 35:453-576 (1981)). Plant growth-promoting rhizobacteria (PGPR) also are able to improve plant growth either through direct effects on the plant (Lugtenberg et al., *Curr. Opin. Biotechnol.* 2:457-464 (1991)) or by suppressing soilborne plant pathogens (O'Sullivan and O'Gara, *Microbiol. Rev.* 56:662-676 (1992); Weller, *Ann. Rev. Phytopathol.* 26:379-407 (1988)). PGPR when applied to soil, seeds or seed pieces, colonize the surface or inside of roots and channels in the rhizosphere that allow physical access to the root. Thus, they are ideally positioned to limit the establishment or spread of pathogens on the roots.

Bacteria introduced for the purpose of suppressing soilborne plant pathogens may interact with pathogens directly through one or more mechanisms of antagonism including competition, parasitism and predation, and antibiosis, or they may function indirectly to limit the initiation or spread of disease by triggering systemic defense responses in the host plant. It has become increasingly clear over the past decade that antibiosis, the inhibition or destruction of one organism by a metabolic product of another, has a dominant role in the control of several important fungal root and seed pathogens by bacterial biocontrol agents, and especially by fluorescent *Pseudomonas* species (Weller and Thomashow, pp. 173-180 in *Pest Management: Biologically Based Technologies*, R. D.

Lumsden and J. L. Vaughn, eds. (1993)).

Pseudomonas is one of comparatively few bacterial genera capable of synthesizing an array of compounds with broad-spectrum antibiotic activity, and many of the most efficient bacterial biocontrol agents are fluorescent *Pseudomonas* strains. Antibiotics produced by strains of fluorescent *Pseudomonas* spp. with biocontrol activity include pyoluteorin and pyrrolnitrin, implicated in control of damping-off diseases of cotton caused by *Pythium ultimum* and *Rhizoctonia solani* (Howell et al., *Phytopathology* 69:480-482 (1979) and *Phytopathology* 70:712-715 (1980)); oomycin A, also involved in suppression of damping-off of cotton caused by *P. ultimum* (Guttererson, *Crit. Rev. Biotechnol.* 10:69-91 (1990)); 2,4-diacetylphloroglucinol, involved in control of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Harrison et al., *Soil Biol. Biochem.* 25:215-221 (1993); Keel et al., *Mol. Plant-Microbe Interact.* 5:4-13 (1992); Vincent et al., *Appl. Environ. Microbiol.* 57:2928-2934 (1991)); and phenazine-1-carboxylic acid and its derivatives, active in suppression of take-all (Thomashow et al., *J. Bacteriol.* 170:3499-3508 (1988); Pierson et al., *Mol. Plant-Microbe Interact.* 5:330-339 (1992)). Of these pathogens, *Gaeumannomyces graminis* and *Rhizoctonia* species are particularly problematic because there are no satisfactory seed treatments for their control. *Rhizoctonia* and *Pythium* species are important because they can infect and causing damping-off and root rot diseases in a wide variety of crop plants. For these reasons, biocontrol agents active against these pathogens are of substantial interest to agriculture.

Whereas most individual biocontrol agents function acceptably only within fairly limited circumstances, biologically active plant-associated microorganisms in the aggregate have almost unlimited genetic biodiversity and are adapted to a wide range of environments. Thus, biocontrol agents that can both antagonize plant pathogens and compete successfully with the indigenous rhizosphere microflora of diverse crops or agroecosystems, are desirable. One proposed approach to obtain such biocontrol agents is to identify genetic elements that can confer or enhance the antifungal activities of rhizosphere colonists indigenous to and highly competitive in the plant and ecological environments where biological control is needed, and use these elements to

genetically engineer strains of biocontrol agents. It is preferred that the biocontrol agents combine the ability to control the growth of one or more fungal pathogens with other desirable attributes such as adaptation to a particular host plant or environment or the ability to rapidly achieve peak growth rates.

The antibiotic 2,4-diacetylphloroglucinol (Phl) is a phenolic compound of possible polyketide origin with antifungal, antibacterial, antiviral, antihelminthic and phytotoxic properties. Phl is produced by fluorescent pseudomonads that suppress root diseases caused by a variety of soilborne plant pathogens of crops around the world. These include root rot of wheat caused by *Fusarium oxysporum*, black root rot of tobacco caused by *Thielaviopsis basicola* (Keel et al., *Symbiosis* 9:327-341 (1990); damping-off of sugar beet caused by *Pythium ultimum* (Fenton et al., *Appl. Environ. Microbiol.* 58:3873-3878 (1992)); damping-off of cotton caused by *P. ultimum* and *Rhizoctonia solani* (Kraus et al., *Phytopathology* 82:264-271 (1992)), blotch of wheat caused by *Septoria tritici* (Levy et al., *Plant Pathol.* 41:335-341 (1992)), and take-all of wheat caused by *Gaeumannomyces graminis* (Harrison et al., supra; Keel et al., (1992) supra; Vincent et al., supra). Strains that produce Phl therefore have considerable agricultural significance.

Three classes of DNA clones have been reported to affect Phl production. The first class contains genes including *gacA* (Laville et al., *Proc. Natl. Acad. Sci. USA* 89:1562-1566 (1992)), *lemA* (PCT Application WO 94/01561; Corbell et al., *Mol. Ecol.* 3:608 (1994)) and *rpoS* (Sarniguet et al., *Mol. Ecol.* 3:607 (1994)), the products of which function as global regulators of a variety of secondary metabolic pathways including that for the synthesis of Phl, thereby indirectly influencing Phl production.

The second class includes DNA sequences of unknown function encoded on the plasmids pME3128 (Keel et al., 1992, supra) and pME3090 (Maurhofer et al., *Phytopathology* 82:190-195 (1992)) from strain CHA0. The former complemented the Tn5 Phl⁻ mutant CHA625 to Phl⁺ and the latter was selected for its ability to cause overproduction of pyoluteorin when introduced into wild-type CHA0; it subsequently was found also to increase Phl production by about 50%. Neither of these loci has been implicated directly in Phl synthesis, nor reported to be able to confer Phl production to

strains deficient in this capacity.

A third class of DNA sequences known to influence Phl production includes those reported by Vincent et al., supra; Fenton et al., supra, and Hara, et al. (Hara et al., pp. 247-249 in *Improving Plant Productivity with Rhizobacteria*, Ryder, Stephens and Bowen, eds. (1994)) that are capable of transferring Phl biosynthetic capability. Vincent et al., supra, described a locus from *P. fluorescens* (formerly *aureofaciens*) Q2-87 (Pierson and Weller, *Phytopathology* 84:940-947 (1994)) that, when disrupted with the transposon Tn5, resulted in the mutant Q2-87::Tn5-1, which was unable to synthesize Phl. Either of two cosmid clones designated pMON5117 and pMON5118 and isolated from genomic DNA of strain Q2-87 restored antifungal activity and Phl production to Q2-87::Tn5-1. Mobilization of pMON5118 into two Phl-nonproducing strains conferred the ability to synthesize Phl and increased their antagonistic activity in vitro against *Gaeumannomyces graminis*, *Pythium ultimum*, and *Rhizoctonia solani*. Vincent et al. did not provide any information as to whether a particular portion of the cloned fragment was required, or if the transferred sequences functioned indirectly as a global regulator or specifically to encode enzymes that catalyze the synthesis of Phl.

Fenton et al., supra, reported that pCU203, containing a 6-kb fragment of DNA cloned from *P. fluorescens* F113, partially restored Phl production to a Phl⁻ Tn5 mutant of F113 and transferred Phl biosynthetic capability only to M114, one of eight nonproducer strains into which it was introduced. Strains F113(pCU203) and M114(pCU203) were more inhibitory to *P. ultimum* in vitro and increased sugarbeet seedling emergence in soil relative to the parental strains. The 6-kb fragment carried monoacetylphloroglucinol transacetylase activity (Shanahan et al., *Anal. Chem.* 272:271-277 (1993)). Fenton et al. did not indicate that a particular portion of the cloned fragment was required, or if the transferred sequences functioned indirectly as a global regulator or specifically to encode enzymes that catalyze the synthesis of Phl. Shanahan et al. likewise did not specify what portion of the 6-kb fragment was required for the transacetylase reaction, nor did they indicate or suggest that the fragment contains genetic information sufficient to encode the full complement of enzymes required to catalyze Phl biosynthesis.

Neither Shanahan et al. nor Fenton et al. have demonstrated that the transacetylase activity is required for or participates in the Phl biosynthetic pathway in F113.

Hara et al., supra, reported that all of eight strains of Phl-nonproducing strains of fluorescent *Pseudomonas* spp., when transformed with the plasmid pPHL5122 containing a 7-kb fragment of DNA from Q2-87, produced Phl, and that the overall severity of take-all was reduced on seedlings of wheat treated with strains that contained the cloned Phl locus as compared to those treated with unmodified parental strains. Hara et al. did not indicate whether a particular portion of the cloned fragment was required, or suggest any particular biological function, e.g., catalytic or regulatory, for the transferred sequences.

SUMMARY OF THE INVENTION

The present invention comprises DNA sequences in isolated and purified form which function specifically in the biosynthesis of 2,4-diacetylphloroglucinol (Phl) and which can effect the production of 2,4-diacetylphloroglucinol (Phl). By effecting production of Phl, is meant that, a DNA sequence of the invention (1) is capable of conferring the ability to produce Phl in a Phl-nonproducing bacterial strain transformed with the sequence; (2) is capable of enhancing Phl production in a Phl-producing bacterial strain transformed with the sequence, or (3) is capable of both conferring and enhancing Phl production.

In effecting production of Phl, a sequence of the invention may function in the biosynthesis of Phl, the regulation of the biosynthesis of Phl, the export of Phl from the producing cell, the modulation of Phl production or activity, or all of the foregoing. Sequences which function specifically in the biosynthesis of Phl include those encoding catalytic enzymes and regulatory proteins specific for the Phl biosynthetic pathway, proteins that export Phl, and sequences that modulate Phl production or activity.

The genetic elements containing the Phl locus are illustrated in FIG. 1, and the sequence data are given in SEQ ID NO: 3. As discussed in detail below, biosynthetic, bioregulator, export, and modulator elements are contained within the locus. The present invention also encompasses sequences shorter than SEQ. ID NO:3

which have the ability to effect Phl production.

The present invention further comprises DNA sequences in isolated and purified form for *phlA*, *phlB*, *phlC*, *phlD*, *phlE*, *phlF*, and *phlR* genes, which genes function in the production of Phl. The aforementioned genes encode proteins, which proteins function in the production (synthesis, regulation of synthesis or modulation) of Phl in bacteria. Genomic sequences encoding particular *phl* gene biosynthetic proteins are specifically exemplified herein. DNA sequences which hybridize specifically to *phl* biosynthetic gene coding sequences or to their components under standard conditions and which encode *phl* gene proteins which function in the biosynthesis of Phl are also encompassed by the present invention.

DNA sequences which function to regulate the biosynthesis of Phl include regulatory and coding sequences which influence expression of the Phl biosynthetic sequences and Phl biosynthetic capability. In particular, a DNA sequence is described which encodes a repressor of Phl biosynthesis, which protein has the function of repressing (stopping) the expression of *phl* biosynthetic genes in bacteria that can otherwise synthesize Phl. Truncation of the repressor sequences resulted in loss of regulation of the *phl* biosynthetic sequences, and overproduction of Phl. The modification of these regulatory and coding sequences to modulate Phl production or to enhance the biocontrol capability of the host bacterial strain is also part of the present invention. Genomic sequences encoding a particular *phl* repressor gene protein, and a truncated derivative thereof, are specifically exemplified herein. DNA sequences that hybridize specifically to the *phl* repressor gene or its complement under standard conditions and which encode repressor genes that function to modulate or repress expression of *phl* biosynthetic genes are included in the present invention.

The present invention further comprises recombinant nucleic acid molecules containing a sequence of the invention. Such molecules include, for example, recombinant vectors, such as cloning, expression, or transformation vectors, which contain a DNA sequence which affects the production of Phl. The invention encompasses recombinant nucleic acid molecules which contain a bacterial regulatory element operably linked to a DNA sequence of

the invention. The bacterial regulatory element may be a promoter from a gene isolated from *Pseudomonas*, *Bacillus*, *Escherichia coli*, or any other bacterium. In one embodiment of the present invention, the bacterial regulatory element is the native promoter of the sequences encoding for the production of Phl. The bacterial regulatory element may be from a gene which is homologous or heterologous to genes of the host bacterial strain.

Another aspect of the invention is the provision of cells which are transformed by the above vectors or DNA sequences.

A particular use of the invention is the provision of bacteria, bacterial biocontrol agents, or bacterial cells transformed with sequences that confer or enhance the ability of the bacteria or bacterial cell to produce Phl. Using the sequences of the invention, biocontrol agents can be produced which combine the ability to control growth of a plant pathogen and to compete aggressively in colonization of the plant rhizosphere. Further, the sequences of the invention contribute to the production of biocontrol agents which are able to inhibit a broad spectrum of plant pathogens.

The present invention also comprises methods of conferring or enhancing Phl biosynthetic capability in a host bacterial strain by transforming the host strain with the recombinant DNA sequences of the present invention. In a particular embodiment of the present invention, the transformed host strain is rendered active or more effective against fungal plant pathogens such as *Gaeumannomyces graminis*, *Rhizoctonia solani*, and species of the genera *Pythium* and *Fusarium*.

The present invention also comprises methods of conferring or enhancing Phl biosynthetic capability in a host bacterial strain by introducing the DNA sequence into the genome of a host bacterial strain. In the preferred embodiments of the invention, the host bacterial strain may be a pseudomonad, particularly strains of the species *Pseudomonas fluorescens*.

The present invention further comprises DNA sequences in isolated and purified form that can be used as probes or oligonucleotide primers for the identification of *phl* genes or functional equivalents thereof in bacterial strains and the use of such probes or primers to isolate DNA sequences encoding *phl* genes or functional equivalents thereof. The DNA sequences that

specifically hybridize to the probes or primers and which encode functional *phl* genes are encompassed by the present invention. Further, because bacterial strains that contain *phl* genes are of potential value as biocontrol agents, the use of any portion of the DNA sequences that confer or enhance Phl biosynthetic capability to identify other strains of bacteria with potential Phl biosynthetic capability is included in the present invention.

Accordingly, it is one object of the present invention to provide DNA sequences and genes that confer or enhance Phl biosynthetic capability in bacterial strains.

It is another object of the present invention to provide gene constructs comprising DNA sequences which have the function of conferring or enhancing Phl biosynthetic capability in bacterial strains.

It is another object of the present invention to provide transformation vectors comprising *phl* gene constructs, which vectors are effective for stably introducing *phl* gene constructs into bacteria.

It is a further object of the invention to provide transgenic bacterial strains wherein the capability to produce Phl has been conferred or enhanced as a result of the introduction of *phl* gene constructs.

It is another object of the invention to provide transgenic bacterial strains enhanced in biocontrol activity against fungal pathogens, wherein the enhancement results from introduction of *phl* gene constructs.

It is a further object of the present invention to provide DNA sequences for use as probes or primers in the identification of strains of bacteria with potential biocontrol activity. Further, DNA sequences and genes are provided as probes and primers for the isolation of homologous genes from related and unrelated hosts. Using such sequences facilitates the identification of genes and the hosts strains harboring them for protection of plants against fungal pathogens.

It is a further object of the present invention to provide DNA sequences and genes which encode proteins that function in the production of Phl, including DNA sequences and genes which encode modified proteins that modulate the production of Phl in bacterial strains. Such modifications may alter the activity of regulatory

genes, the expression of *phl* genes encoding biosynthetic proteins, and the biocontrol activity of host bacterial strains.

According to the present invention, the above objectives may be carried out by the isolation and use of DNA sequences that confer or enhance the production of 2,4-diacetylphloroglucinol in bacterial strains. The isolation of these genes and associated regulatory sequences is important for several reasons. First, in bacterial strains they confer or enhance the production of Phl, which is able to inhibit a wide range of fungal plant pathogens including *Gaeumannomyces graminis*, *Rhizoctonia solani*, and species of the genera *Pythium* and *Fusarium*. Secondly, they can function as probes and primers to screen for and identify naturally-occurring strains of bacteria with Phl biosynthetic capability. The use of bacterial strains transformed with DNA sequences that confer or enhance the production of Phl, or of Phl-producing strains identified by use of probes or primers derived from such sequences, provides an environmentally safe and effective method for control of these pathogens.

Examples of the sequences conferring Phl biosynthetic capability of the present have been deposited. Accordingly, the sequences conferring or enhancing Phl biosynthetic capability include the exemplified or deposited DNA sequences as well as the fragments derived from the exemplified or deposited sequences. By fragment is intended a DNA sequence which is capable of functioning to confer or modulate Phl biosynthetic capability or to identify the potential for such capability in bacteria.

The present invention further includes portions of such DNA fragments which function specifically in the synthesis of Phl but may not in themselves be sufficient to effect the production of PHL in transformed cells.

Other objects and advantages of this invention will become readily apparent from the ensuing description.

DEFINITIONS

As used in the present application, the following terms have the meaning set out below:

Promoter or regulator DNA sequence: An untranslated DNA sequence which assists in, enhances, or otherwise affects the transcription, translation, or expression of an associated

structural DNA sequence which codes for a protein or other DNA product. The promoter DNA sequence is usually located at the 5' end of a translated DNA sequence, typically between 20 and 100 nucleotides to the 5' end of the starting site for translation.

Structural or coding DNA sequence: A DNA sequence that is translated or transcribed in an organism to produce an RNA, a protein, or other DNA product.

Associated with/operably linked: Two DNA sequences which are "associated" or "operably linked" are related physically or functionally. For example, a promoter or regulator DNA sequence is said to be "associated with" a DNA sequence that codes for an RNA or a protein if the two sequences are operably linked, or situated such that the regulator DNA sequence will affect the expression level of the coding or structural DNA sequence.

Derived from: A first DNA sequence or fragment is said to be "derived from" a second DNA sequence or fragment if the former is physically isolated from the latter, or if the former is isolated by using part or all of the latter as a probe for isolation.

Substantial sequence homology: A sequence having substantial functional and structural equivalence between sequences of nucleotides or amino acids. In the case of a nucleotide sequence, a sequence having substantial sequence homology will have at least 70%, preferably 85%, and more preferably 90% homology to a reference nucleotide sequence, as opposed to nonspecific binding, and function specifically in the biosynthesis of Phl. Homologous sequences can be identified in a hybridization experiment. Conditions of high and low stringency are described below. In the case of amino acid sequences, the different sequences have at least 70%, more preferably 80%, and most preferably 90% or more similarity between the polypeptides coded for by the amino acid sequences and function specifically in the biosynthesis of Phl.

Locus: A segment of DNA that includes one or more coding or regulator DNA sequences that specifically affect a particular phenotypic trait.

Transformed cell: A cell or an ancestor of a cell that contains DNA introduced by the hand by any means known to the art, including but not limited to transformation, conjugation, and electroporation.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. This figure shows the genetic organization of the DNA sequences present in SEQ ID NO:3. Names in italics represent open reading frames (genes), whereas names within boxes represent the predicted protein sequences. The gray vertical arrow identifies the site of insertion of the Tn5 transposon in Q2-87::Tn5-1. The line at the bottom marked by Phl shows the approximate location of the segment of DNA included in SEQ ID NO:4, and capable of conferring or enhancing Phl production in transformed strains of *Pseudomonas*. The arrows marked Phl⁻ delineate the position and orientation of sequences identified by Tn3HoHol mutagenesis as being essential for Phl biosynthesis. The arrow marked Red⁻ represents the region within which insertions caused loss of red pigment production and reduced Phl production. Restriction enzyme sites: B=BamHI, C=ClaI, E=EcoRV, H=HindIII, P=PstI, R=EcoRI, S=SalI.

Figure 2. This figure indicates the location of Tn3HoHol insertions used to define sequences required to confer or enhance Phl biosynthetic capability in the plasmid pMON5122. Filled and open circles represent Lac⁺ and Lac⁻ insertions respectively. Lac⁺ insertions are transcriptionally active, whereas Lac⁻ insertions are not. Insertions are numbered sequentially from right to left except for insertions 40, 41, 42, and 43 which are marked. Horizontal arrows marked Phl⁻ indicate the regions in which insertions caused loss of Phl biosynthetic capability. The arrow marked Red⁻ represents the region within which insertions caused loss of red pigment production and reduced Phl production. A vertical arrow indicates the position of the Tn5 insertion in Q2-87::Tn5-1. Restriction sites: B=BamHI, Bs=BstEII, C=ClaI, E=EcoRV, H=HindIII, P=PstI, Pv=PvuII, S=SalI.

Figure 3. Subclones generated for determination of the DNA sequences given in SEQ ID NO:3. Restriction enzyme sites used to generate the subclones were: 5124, BamHI, ClaI; 5125, PstI, ClaI; 5126, ClaI, EcoRI; 5127, SalI, EcoRV; 5128, ClaI; 5129, ClaI; 5130, EcoRV, ClaI; 5131, ClaI, EcoRV; 5132, SalI, EcoRI; 5133, SalI; 5134, BamHI. Horizontal arrows marked Phl⁻ indicate the regions in which transposon insertions caused loss of Phl biosynthetic capability. The arrow marked Red⁻ represents the region within which insertions caused loss of red pigment

production and reduced Phl production. The vertical arrow identifies the site of insertion of the Tn5 transposon in Q2-87::Tn5-1. Restriction enzyme sites: B=BamHI, C=ClaI, E=EcoRV, H=HindIII, P=PstI, R=EcoRI, S=Sall.

Figure 4. Proposed biosynthetic pathway for 2,4-diacetylphloroglucinol, adapted from D. E. Metzler, *BIOCHEMISTRY, The Chemical Reactions of Living Cells*. Chain building is thought to be initiated by the condensation of an acyl thioester "starter" unit with a malonyl "extender" unit to yield CO₂ plus a ₄C acetoacetyl intermediate which is then further elongated by sequential condensation reactions with additional acetyl groups donated by malonyl extender units. Monoacetylacetylphloroglucinol (MAPG) could then be formed as shown. The conversion of MAPG to 2,4-diacetylphloroglucinol (Phl) by an enzyme designated MAPG acetyltransferase has been demonstrated *in vitro* by *P. fluorescens* F113 by Shanahan et al. Whether Phl also may arise directly by cyclization of a ketodecanoyl thioester intermediate is not known.

Figure 5. Similarity of PhlC to the thiolase portion of sterol carrier protein x (SCPx). Boxed region at the left end of PhlC indicates the position of the conserved putative substrate-binding site; oval represents conserved glycine-rich C-terminal region.

Figure 6. Conservation in PhlD of the active site residues and the signature sequence of the chalcone synthase family. The asterisk marks the catalytic cysteine.

Figure 7. Kyte-Doolittle hydropathy plots of PhlE and *Staphylococcus norA* protein. Both show central hydrophilic loops flanked by hydrophobic, potential membrane-spanning regions of 19 or 21 amino acids (Window size = 19, 21).

Figure 8. Conserved helix-turn-helix motif of the repressor protein PhlF as compared to similar domains in other known repressor proteins including the λ cro and λ repressor proteins, phage 434 cro and repressor proteins, lac repressor, catabolite reactive protein (CRP), and *E. coli* tetracycline repressor protein. The HTH score is a measure of probability that the amino acid sequence predicts a functional DNA-binding domain and was higher for the PhlF protein than for the known λ cro and λ repressor proteins as well as the phage 434 cro and repressor proteins.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides DNA sequences in isolated and purified form which function specifically in the biosynthesis of 2,4-diacetylphloroglucinol (Phl) and which can effect the production of Phl by conferring or enhancing the ability of an organism to produce Phl. Manipulation of these genetic elements, either separately or in combination, can be used to introduce or enhance biosynthetic capability in organisms transformed with the sequences.

In particular, we have identified, cloned, and sequenced DNA sequences from *Pseudomonas fluorescens* Q2-87 and used the sequences to introduce Phl biosynthetic capability in Phl-nonproducing bacterial strains. We also have determined that strains of *Pseudomonas* capable of producing Phl, when transformed with these sequences, are enhanced in Phl production. Further, bacterial biocontrol agents transformed with these sequences produce Phl and are enhanced in their biocontrol activity.

The present disclosure is the first report of the cloning, sequencing and reproducible mediation of transgenic Phl production by *phl* genes of *Pseudomonas*.

The *phl* genes include genomic sequences which encode *phl* gene proteins and coding and regulatory sequences which direct and regulate the transcriptional and translational expression of the *phl*-coding sequences. As defined herein, "*phl* gene proteins" refers to proteins having the ability to function in the production of 2,4-diacetylphloroglucinol in bacteria synthesizing *phl* gene proteins. The *phl* locus is that segment of DNA that encompasses the coding and associated regulator DNA sequences that specifically encode the ability to confer or enhance Phl production. Exemplified *phl* gene products have predicted molecular masses of 27,679 kDa (PhlA); 27,445 kDa (PhlB); 41,721 kDa (PhlC); 38,389 kDa (PhlD); 45,259 kDa (PhlE); 23,044 kDa (PhlF) and 45,473 kDa (PhlR). The predicted amino acid sequence of PhlA is given in SEQ ID NO:6. The predicted amino acid sequence of PhlB is given in SEQ ID NO:8. The predicted amino acid sequence of PhlC is given in SEQ ID NO:10. The predicted amino acid sequence of PhlD is given in SEQ ID NO:12. The predicted amino acid sequence of PhlE is given in SEQ ID NO:14. The predicted amino acid sequence of PhlF is given in SEQ ID

NO:16. The predicted amino acid sequence of PhlR is given in SEQ ID NO:18. The predicted amino acid sequence of a truncated PhlF protein is given in SEQ ID NO:20. The exemplified genomic DNA sequence which encompasses regulatory and coding sequences for these *phl* gene products is provided in SEQ ID NO:3. A truncated genomic DNA sequence (from pMON5122) is given in SEQ ID NO:4.

The degeneracy of the genetic code is well known to the art; therefore, synonymous coding sequences with one or more codon substitutions can be readily determined by one of ordinary skill in the art. Synonymous coding sequences vary from the exemplified coding sequences but encode proteins of the same amino acid sequences as those specifically provided herein.

Specific embodiments of nucleotide sequences which encode *phl* gene proteins which have the function of participating in the synthesis of Phl are given in SEQ ID NOS:5 (*phlA*), 7 (*phlB*), 9 (*phlC*), 11 (*phlD*), and 17 (*phlR*). A nucleic acid sequence that encodes a *phl* gene protein that functions to regulate the expression of *phl* genes and the production of Phl is given in SEQ ID NO:15 (*phlF*). A truncated version of this sequence which encodes a truncated *phl* gene protein that facilitates overproduction of Phl is given in SEQ ID NO:19. A nucleic acid sequence that encodes a *phl* gene protein that functions to enhance the antagonistic activity of Phl-producing bacteria transformed with *phl* genes is given in SEQ ID NO:13 (*phlE*).

A genomic DNA sequence containing *phl* genes required for Phl synthesis is presented in SEQ ID NO:1. The DNA sequence is 3680 bp in length. Nucleotide sequence analysis reveals five open reading frames (coding portions) and intergenic spaces that initiate at base 2 and terminate at base 3680 (Figure 1). The open reading frame designated *phlA* initiates at base 3680, terminates at base 2889 and encodes a protein, PhlA, 264 amino acids in length. The open reading frame designated *phlB* initiates at base 1917, terminates at base 1210, and encodes a protein, PhlB, 236 amino acids in length. The open reading frame designated *phlC* initiates at base 2855, terminates at base 1689, and encodes a protein, PhlC, 389 amino acids in length. The open reading frame designated *phlD* initiates at base 1048, terminates at base 2, and encodes a protein, PhlD, 349 amino acids in length. The open reading frame designated *phlR* initiates at base 2118,

terminates at base 3371, and encodes a protein, PhlR, of 418 amino acids. The predicted proteins PhlA, PhlB, PhlC, PhlD and PhlR are described in Example 4, below.

A genomic DNA sequence containing *phl* genes that function in Phl production is presented in SEQ ID NO:2. The DNA sequence is 5076 bp in length. The resulting open reading frames and intergenic spaces initiate at base 2 and terminate at base 5076. This sequence encompasses that of SEQ ID NO:1 and 1396 contiguous bases which include an open reading frame initiating at base 1270 and terminating at base 2. This open reading frame encodes a protein, PhlE, 423 amino acids in length. The encoded protein is described in Example 4, below.

A genomic DNA sequence containing *phl* genes that function in Phl production is presented in SEQ ID NO:3. The DNA sequence is 6387 bp in length. The resulting open reading frames, intergenic spaces and regulatory regions initiate at base 2 and terminate at base 6387. This sequence encompasses that of SEQ ID NO:2 and 1311 contiguous bases which include regulatory sequences and an open reading frame initiating at base 5781 and terminating at base 6386. This open reading frame encodes a protein, PhlF, 202 amino acids in length. The encoded protein is described in Example 4, below. The DNA sequence of SEQ ID NO:3 is contained within a *SalI*-*BamHI* fragment of 7203 base pairs in the clone pMON5120, which was deposited under terms of the Budapest Treaty June 21, 1995, with the USDA Agricultural Research Service Culture Collection, Northern Regional Research Laboratory (NRRL), and has been designated NRRL accession number B-21477.

A genomic DNA sequence which contains *phlACBDE*, *phlR*, and a truncated *phlF* gene relative to that of SEQ ID NO:3 and SEQ ID NO:15 is given in SEQ ID NO:4. This genomic DNA is 6170 bp in length. Open reading frames, intergenic spaces and regulatory regions initiate at base 2 and terminate at base 6170. This sequence encompasses that of SEQ ID NO:2 and 1094 contiguous bases which include regulatory sequences and a portion of the *phlF* open reading frame initiating at base 5781 and terminating at base 6170. The truncated *phlF* protein is 130 amino acids in length. Its DNA coding and amino acid sequences are given in SEQ ID NO:19 and SEQ ID NO:20, respectively. When transformed into bacterial strains, the sequences present in SEQ ID NO:4 confer or enhance

Phl production. The DNA sequence of SEQ ID NO:4 is contained within a *SalI*-*EcoRI* fragment of 6586 bp in the plasmid pPHL5122, which was deposited under terms of the Budapest Treaty June 21, 1995, in the Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois, and has been designated NRRL accession number B-21476. Nucleotide and amino acid sequences having substantial sequence homology to the exemplified or deposited sequences are encompassed in this invention.

Sequences for Phl Production

Genomic sequences capable of inducing Phl production may transfer either gene(s) that encode biosynthetic enzymes or a positive regulatory element that can activate the expression of unassociated and unexpressed (cryptic) genes indigenous to the host strain. Examples of cryptic genes that have been activated by introduction of positive regulatory elements include those for the synthesis of Phl and HCN. Mutations in genes encoding positive regulators typically have a complex phenotype because the functional gene product is unavailable to activate the expression of other genes, including those for the production of Phl and HCN. We found no evidence that Phl production in the transformed strains could be explained by introduction of a positive global activator of gene expression.

We have found that DNA sequences encompassing production of Phl are contained within a DNA fragment encompassing five genes, *phlACBD* and *phlR* (Figure 1), and that the coding sequences of these genes predict proteins with catalytic activities consistent with roles in the biosynthesis of Phl.

Of the DNA fragments from *P. fluorescens* Q2-87 implicated by Vincent et al. in the production of the Phl, only the fragment designated pMON5122 was able to transfer Phl biosynthetic capability to the Phl-nonproducing strain *P. fluorescens* M4-80R (Example 1; Table 1). Fragments smaller than that in pMON5122 were unable to transfer Phl biosynthetic activity, suggesting that essential sequences had been deleted, and fragments larger than pMON5122 also failed to confer biosynthetic capability, suggesting that they contained negative regulator sequences.

The cloned fragment from pMON5122 was transferred to the plasmid pVSP41, a vector which is stably maintained in *Pseudomonas* in the absence of antibiotic selection, and introduced into five

Phl-nonproducing strains and into Q2-87. Derivatives of all of Phl-nonproducing strains containing pPHL5122 produced Phl but were unaltered in other phenotypes including production of HCN. Phl production by Q2-87(pPHL5122) was enhanced over that by Q2-87 alone or that by Q2-87(pVSP41), the vector-bearing strain (Example 1, Table 2).

Q2-87::Tn5-1, shown by Vincent, et al. to be defective in Phl production, contains the transposon Tn5 within a genomic copy of the sequences cloned on pPHL5122. Phenotypic characterization Q2-87::Tn5-1 revealed that it remained HCN⁺.

Field studies of strains containing the cloned sequences that confer or enhance Phl production will require that introduced genes be stably incorporated into the chromosome of the host strain. As illustrated in Example 1, below, we used the mini-transposon vector pUT mini-Tn5 Km (de Lorenzo and Timmis, *Meth. in Enzymol.* 235:386-405 (1994)) to transfer sequences from pMON5122 to the chromosomes of rhizosphere pseudomonads including strains Q69c-80, Q65c-80, and the phenazine antibiotic-producing strains 2-79 and 30-84. Other materials for stable introduction are known to those in the art and are encompassed by this invention.

To confirm insertion of the mini-Tn5 vector, the kanamycin-resistant transposition derivatives of Q69c-80 were characterized. Results from Southern hybridization indicated that single copies of the Phl genes had transposed to different sites in individual derivatives, and all of the derivatives initially produced Phl in vitro. Quantities of Phl sometimes were over twenty-fold greater than produced by Q69c-80(pPHL5122) (Example 1; Table 3), and Phl usually was not detectable in cultures of strain Q2-87 grown under comparable conditions.

The reproducibility of transfer and the specificity of the affected phenotype in these studies indicate that sequences present on pMON5122 include genes encoding a Phl biosynthetic pathway, rather than a global activator of Phl synthesis.

The portion of pMON5122 active in Phl production was defined by mutagenesis with the transposon Tn3HoHo1 (Stachel et al., *EMBO J.* 4:891-898.(1985)). The site and orientation of each insertion were determined (Example 2; Figure 2), and the effect on Phl production and expression of the transposon-encoded *lacZ* gene were evaluated after introduction of the mutated plasmids into the Phl-

nonproducing strain M4-80R. All insertions which resulted in expression of *lacZ*, except three, were similarly oriented, only the Lac⁺ insertions 2 and 3 at the right end and insertion 38 at the left end of the cloned fragment had the opposite orientation (Example 3; Figure 2).

Insertions resulting in reduction or loss of Phl production, spanned approximately 5 kb of the 6.5 kb fragment and delineated at least two similarly oriented transcriptional/translational units (Example 2; Figure 2, Table 4). These results show that not all of the sequences present on pMON5122 are necessary for Phl production.

As shown in Example 2 below, whereas all insertions up to and including number 18 that were Phl⁻ also failed to produce the red pigment, insertions downstream of number 18 exhibited different phenotypes. Most of these insertions had no effect on Phl synthesis (Table 4) and transformants containing the mutated plasmids had the characteristic red color. However, insertion derivatives 20, 25, and 33 exhibited a level of Phl production that was intermediate between the positive and negative controls, remained inhibitory to *C. michiganensis* subsp. *michiganensis* in vitro (Table 4), and failed to exhibit red pigmentation. The region containing insertions 20, 25 and 33 is designated Red⁻ in Figures 1 and 2 and is considered to provide a function that enhances, but is not required for Phl production in M4-80R.

Subclones spanning the ca. 5-kb region were generated (Figure 3) and submitted to the Nucleic Acid Sequencing Facility, Iowa State University, for sequence determination. Using the Genetics Computer Group (GCG) package as supported by the Washington State University VADMS facility, we have identified six large complete open reading frames, designated *phlA*, *phlB*, *phlC*, *phlD*, *phlE*, and (in the opposite orientation) *phlR* within this region (Figure 1). The fragment containing *phlA* through *phlD* encoded on one DNA strand, with *phlR* on the other strand, contains genes necessary to confer or enhance Phl production, and *phlD* is required for the synthesis of Phl. A larger fragment encompassing the above plus the adjacent *phlE* gene can confer or enhance Phl production and includes an additional gene, the product of which enhances the synthesis, activity, and/or export of Phl. The function of this gene is not required for Phl production in transformed M4-80R, but

such function may be necessary for Phl production or export in other bacterial host strains.

DNA sequences for production of Phl encode catalytic proteins

The enzymatic steps required for Phl synthesis in bacteria are unknown, but can be anticipated based on knowledge that the structure of Phl is consistent with synthesis via a polyketide mechanism (Figure 4). Similarities also are known to exist between fatty acid and polyketide synthases, β -ketoacyl synthases (FAS), thiolases, and polyketide synthases (PKS) in bacteria, and the stilbene and chalcone synthase (STS/CHS) enzymes from plants. Synthesis of both polyketides and fatty acids is known to initiate by condensation of an acetyl starter unit with malonyl-ACP; this step generally is catalyzed by a β -ketoacyl synthase separate from the one(s) that catalyze subsequent chain elongation via condensation reactions that sequentially add C₂ units from malonyl thioesters to the growing chain. In bacterial FAS, separate acetyl and malonyl transferases are required, as is a transferase for chain termination. PKS in *Streptomyces* resembles FAS in that ACP-linked residues are implicated as building units based on the presence in PKS loci of an ACP gene. A significant difference between CHS and the FAS and PKS β -ketoacyl synthases is that CHS requires neither ACP nor specific transferases; malonyl-CoA units are added to a coumaroyl-CoA starter unit. Based on these precedents, it is reasonable that Phl synthesis minimally will require a chain-initiating β -ketoacyl synthase; a malonyl condensing enzyme, and a cyclase for ring closure. Depending on the mechanism of transfer, and acyl carrier protein may also be required.

Analysis of the protein sequences encoded by SEQ ID NO:1 and comparison to other protein sequences revealed conserved structural motifs and similarities to known proteins that are consistent with predicted functions in Phl biosynthesis via the polyketide pathway. Motifs conserved (mismatch=1) within PhlA include the short chain alcohol dehydrogenase family signature, the ornithine/diaminopimelic acid/arginine decarboxylase family 2 signature, and a phosphopantetheine attachment site (Example 4). These motifs suggest that PhlA may function either in the provision of substrates for PhlD and/or as an acyl carrier.

A partially conserved (mismatch=1) thiolase signature sequence within the PhlR protein sequence indicates similarity to type II thiolases that catalyze the thiolysis of acetoacetyl-coA and are involved in pathways such as poly- β -hydroxybutyrate synthesis and steroid biogenesis which resemble the Phl pathway (Figure 4) in their dependence on condensation reactions involving short-chain acyl thioesters.

Analysis of the PhlC protein sequence and comparison to other protein sequences revealed significant similarity to the N-terminal portion of mammalian peroxisomal sterol carrier protein X (SCPx) (Example 4; Figure 5). This portion of SCPx has 3-oxoacyl-CoA thiolase activity, and is hypothesized to supply acetyl-CoA for anabolic reactions in cells well-supplied with energy. By analogy to SCPx, the PhlC product may provide a source of CoA starter units for the synthesis of Phl. Alternatively, because thiolases share conserved residues in their condensing domains with β -ketoacyl synthases and chalcone synthases, the similarity we have detected between PhlC and SCPx may indicate activity in chain elongation reactions analogous to those that occur in fatty acid and polyketide biosynthesis.

PhlD is required for Phl production, and the PhlD protein revealed a highly significant similarity to members of the chalcone synthase/stilbene synthase (CHS/STS) family of enzymes from higher plants. PhlD contained regions with extensive similarity to the active site and signature sequence domains of CHS/STS enzymes (Example 4; Figure 6) but had surprisingly little similarity to prokaryotic β -ketoacyl synthases, including those that catalyze similar condensing reactions (e.g., polyketide synthesis by actinomycetes) in bacteria. It is, to our knowledge, the first prokaryotic protein to show such extensive similarity to the CHS/STS protein family. Acylphloroglucinols have been recovered as by-products of chalcone synthesis *in vitro*, suggesting that PhlD may be sufficient to catalyze the condensation and/or cyclization reactions required for synthesis of monoacetylphloroglucinol (Figure 4).

Based on results from mutagenesis (Example 2), phenotypic characterization of transformants (Example 1) containing DNA fragments that confer or enhance Phl biosynthetic capability, and sequence analysis of the predicted protein sequences encoded

within these fragments (Example 4), the segment of DNA containing *phlACBD* and *phlR* comprises the minimum known to date to be required for production of Phl.

Contiguous sequences modulate Phl production

Mutagenesis of pMON5122 with Tn3HoHo1 revealed a locus designated Red and located adjacent to those sequences present in SEQ ID NO:1. The Red locus was not itself required for Phl production, but M480-R(pMON5122), containing certain mutations in Red were less inhibitory in vitro (Table 4). The Red locus therefore had the capacity to enhance Phl production or activity, perhaps by facilitating Phl export, in strains transformed with a DNA segment that includes *phlACBD* and *phlR*.

The DNA sequences within the Red locus were found to contain an open reading frame encoding a protein designated PhlE with significant similarity to *Staphylococcus aureus* *norA* protein, which contributes to fluoroquinone resistance. NorA and other such proteins are members of a very large superfamily of transmembrane solute facilitators; among the most well-known representatives of the drug efflux subfamily are the tetracycline resistance proteins. PhlE retained conserved structural features of these integral membrane permeases (Figure 7), including a central hydrophilic loop bordered on either side by six hydrophobic α -helices. The results of phenotypic analysis of mutations in the Red region, combined with knowledge of the predicted structure of the PhlE protein are consistent with PhlE functioning in the export of Phl. Such export would reduce the intracellular Phl concentration, and may represent a mechanism of resistance by which bacteria producing Phl avoid autointoxication.

A seventh open reading frame designated *phlF* was identified upstream of and oriented divergently from *phlABCDE*. This open reading frame is present in truncated form in pPHL5122. The DNA sequences including *phlABCDE*, *phlR*, the intervening sequences (which are likely to contain promoters based on their position and AT-richness) and the truncated *phlF* gene are included in SEQ ID NO:4. In pPHL5122 this DNA fragment was capable of conferring or enhancing Phl production in all strains of *Pseudomonas* into which it was introduced (Example 1). The DNA sequence consisting of *phlABCDE*, *phlR*, the intervening sequences containing the putative

promoter region, and the complete *phlF* gene are given in SEQ ID NO:3.

The PhlF protein sequence was similar to those of tetracycline repressor protein, and other known repressors, particularly within a conserved helix-turn-helix domain (Figure 8) which is a characteristic feature of such DNA-binding repressors. It is known that the tetracycline repressor controls expression of the *terR* resistance gene, and that repressor gene deletions resulting in truncation of the carboxy-terminal end of the repressor protein can result in overexpression of the resistance protein. By analogy, the very high levels of Phl production by transformed derivatives of *P. fluorescens* Q69c-80 containing a construct consisting of the truncated *phlF* and the *phlACBDE* and *phlR* genes (Table 3) support the argument that the *phlF* gene is a repressor of *phl* gene expression. That pMON5120, containing an intact *phlF* gene, does not transfer Phl biosynthetic capability (Table 1) further strengthens the argument.

Those skilled in the art will also be aware that it will be possible to improve the effectiveness of a Phl-producing biological control strain by modulating the level of expression or activity of that strain's Phl biosynthetic genes. This can be accomplished by genetic manipulation of cloned genes such as *phlE* and *phlF*, followed by introduction into the genome. In strains that already produce Phl, the altered genes may be introduced to replace the native genes by homologous recombination, or may be introduced at a separate location. An example of the latter is the introduction by transposition (according to the method given in Example 1) of a heterologous promoter operably linked to the truncated *phlF* gene. The truncated PhlF protein competes with the intact protein, relieving the repression caused by the native protein and permitting increased Phl production. The level of competition, and hence, the level of Phl overproduction, is controlled by the strength of the heterologous promoter relative to the native *phlF* promoter. Such promoters are readily available to those skilled in the art, and a possible means of fusing them to genes such as the truncated *phlF* gene, involves the use of the overlap extension polymerase chain reaction strategy (Horton et al., *Gene* 77:61 (1989)). Alternatively, the truncated *phlF* gene, under control of its native promoter or a heterologous promoter,

is substituted by homologous recombination for the genomic *phlF* gene, again effecting increased Phl production.

Another aspect of the invention is genetically engineered recombinant nucleic acid molecules, i.e., non-naturally occurring nucleic acid molecules, preferably DNA, containing a portion encoding a *phl* gene protein or a functional *phl* gene homolog, which has the function of conferring or enhancing Phl production in bacteria expressing *phl* genes or functional homologs thereof. A recombinant DNA molecule refers to a hybrid DNA sequence comprising at least two DNA sequences, the first sequence not normally being found together in nature with the second. Such molecules can be obtained by manipulation of genetic material using restriction enzymes, ligases, and similar recombinant techniques as described by, for example, Sambrook et al., (*Molecular Cloning*, second edition, Cold Spring Harbor Laboratory, Plainview, NY; (1989)), Ausubel et al., *Current Protocols in Molecular Biology*, Current Protocols (1989), and *DNA Cloning: A Practical Approach*, Volumes I and II (ed. D. N. Glover) IREL Press, Oxford, 1985. Examples include recombinant vectors, such as cloning or expression vectors which contain DNA sequences encoding *phl* gene proteins which are in a 5' to 3' (sense) orientation or in a 3' to 5' (antisense) orientation. "Recombinant," as used in the present application, does not refer to naturally-occurring genetic recombinations.

The recombinant DNA sequences of the present invention may originate from *Pseudomonas* or from organisms of a different taxonomic classification. The recombinant DNA sequences of the present invention may further comprise one or more regulatory DNA sequences operably linked to the structural DNA sequences above. Such regulatory sequences include promoter sequences, leader sequences, and other DNA sequences which may affect the expression of the regulatory DNA sequences, as well as those fragments of a regulator DNA sequence that are able to act with such effect. Bacterial regulatory elements known in the art include any bacterial regulatory element that is known to function as a promoter, enhancer, ribosome binding site, and/or any other regulatory control mechanism of the associated coding sequence. Bacterial regulatory elements include hybrid regulatory regions comprising mixtures of parts of regulatory elements from different

sources. For example the *trp-lac* (*tac*) promoter combines the -35 region of the *E. coli* tryptophan operon with the -10 region of the *E. coli lac* operon promoter and functions effectively in *Pseudomonas* (Bagdasarian et al., Gene 26:273-282 (1983)). An associated coding DNA sequence is one that is adjacent or adjoining 3' to the regulatory elements and which codes for a protein when transcribed and translated. Bacterial regulatory elements can be isolated from commercially available vectors, bacterial regulatory elements known in the art, and bacterial regulatory elements identified by using promoterless marker-containing transposons or promoter selection vectors.

As used herein, a nucleic acid molecule can be a DNA molecule, an RNA molecule or a DNA-RNA hybrid molecule. A non-naturally occurring nucleic acid molecule is one which does not occur in nature. A non-naturally-occurring nucleic acid molecule, includes, for example, DNA sequences, in isolated and purified form; a recombinant nucleic acid molecule having a heterologous region, that is, an identifiable segment of DNA that is not covalently linked to the *Phl* gene coding sequences in nature; or such a non-naturally occurring molecule may be constructed of parts which have been chemically synthesized; or a synthetic sequence having codons different than the native gene. Parts from heterologous sources may be joined by any means known to the art, e.g., by ligation *in vitro*. Alternatively, parts may be joined in an *in vivo* process such as by recombination, but such recombination will be directed by the hand of man and the desired result will be identified by man.

The Use of *Phl* Sequences for Plant Pathogen Control

It is an aspect of the present invention that improved biological control strains can be generated following the introduction of genes that confer *Phl* biosynthetic capability into a variety of bacterial isolates from nature. This approach represents a method for the generation of potentially effective biocontrol strains adapted to a variety of crop plants or ecosystems.

It is a further aspect of the present invention that *Phl*-producing biological control strains can be rendered more effective by the introduction of genes described herein that

enhance Phl biosynthetic capability.

In another embodiment of the present invention, biocontrol agents are provided which are able to inhibit the growth of fungal pathogens, such as *Gaeumannomyces graminis*, *Rhizoctonia solani*, and species of the genera *Pythium* and *Fusarium*. These biocontrol agents may be bacteria, plant cells or animal cells transformed with the recombinant DNA sequences above, but are preferably bacterial strains, and more preferably Gram negative bacteria, such as those of the genus *Pseudomonas*. Most preferred as biocontrol agents are strains of the species *Pseudomonas fluorescens*.

Another embodiment of the present invention provides methods of inhibiting the growth of fungal pathogens, such as *Gaeumannomyces graminis*, *Rhizoctonia solani*, and species of the genera *Pythium* and *Fusarium*. In the methods of the present invention, the DNA sequences that confer or enhance Phl production can be introduced into the genome of a bacterial strain which may not ordinarily be effective as an inhibitor of fungal pathogens, resulting in an effective biocontrol strain.

DNA in the form of plasmids can be transferred from one bacterium to another by a sexual process termed conjugation. Plasmids that mediate conjugal transfer contain genes that code for the synthesis of pili. These pili are hollow filaments that join the plasmid-bearing (donor) bacterium with another bacterium (recipient), and through which replicated copies of the plasmid pass from the donor to the recipient. This procedure occurs naturally and is utilized in the laboratory as a method of transferring genes between bacteria. For some strains of *Pseudomonas*, conjugal transfer is the preferred method of transfer because these bacteria are not readily transformed with isolated and purified DNA.

Most of the plasmids commonly used as vectors for conjugal transfer in the laboratory do not themselves cause conjugation, but are transmissible and can be mobilized when conjugation is mediated by a coresident conjugative plasmid. Transmissible plasmids may have a broad host range (i.e., able to replicate in a variety of bacterial hosts) or a narrow host range (i.e., replication is limited to *E. coli*). Examples of broad host range transmissible plasmids suitable for genetic analysis in Gram-

negative soil bacteria are provided in *Transformation of Plants and Soil Microorganisms*, K. Wang, A. Herrero-Estrella, and M. Van Montagu, Eds., Cambridge University Press, Cambridge (1995). Broad host range transmissible plasmids may be stably maintained in *Pseudomonas* in the absence of selection (e.g., pPHL5122) or more usually maintained only in the presence of an antibiotic, resistance to which is encoded on the plasmid (e.g., pMON5122).

Plasmids that are stably maintained under selection are suitable as vectors *in vitro* (Tables 1 and 2), but are unacceptable when the cloned DNA they carry must be functionally evaluated in an environment such as soil where it is not possible to maintain continuous antibiotic selection. Stably maintained transmissible plasmids are appropriate for use as vectors under contained conditions such as the growth chamber where persistence of the plasmid in the host is desired, and there is no danger of gene escape into the environment (Table 5). Bacteria harboring transmissible vectors containing cloned DNA may not be released into natural environments, however, because the possibility exists for uncontrolled mobilization of recombinant DNA mediated by conjugative plasmids indigenous to native soil microorganisms.

Stable insertion of cloned *phl* genes into bacterial strains in a manner suitable for environmental release may be accomplished by homologous recombination or by transposition. When mediated by recombination, a selectable marker is first introduced into or adjacent to the *Phl* DNA in such a way that *phl* gene functions are not disrupted. The marked *phl* genes are cloned into a fragment of chromosomal DNA containing the target site of insertion such that the sequences containing the marker gene and the *phl* genes are flanked on either side by target site sequences of no less than 500 bp, and preferably of at least 1 kb. The entire recombinant fragment is introduced into the target strain on a transmissible plasmid, and transformants are selected that retain the *Phl* gene marker, but not that of the vector.

Transposons provide an alternative means to the generation of stable chromosomal insertions. Sequences containing *phl* genes are cloned within a transposable element which contains a selectable marker and is carried on a transmissible plasmid. Transposition functions are provided by genes encoded on a plasmid not maintained in the target strain; this may be the plasmid carrying

the transposable element (Herrero et al., *J. Bacteriol.* 172:6557-6567 (1990); de Lorenzo et al., *supra*) or a separate plasmid (Barry, *Gene* 71:75-84 (1988)). The construction of a transposable element containing *phl* genes is described in Example 1, and an example of its use in *in situ* assessment of *phl* gene activity is given in Example 8.

In another embodiment of the present invention, methods are provided for producing Phl which is effective in inhibiting the growth of fungal pathogens including *Gaeumannomyces graminis*, *Rhizoctonia solani*, and species of the genera *Pythium* and *Fusarium*. This method comprises introducing the recombinant sequences of the present invention into the genome of a bacterium, allowing the transformed bacteria to produce Phl, and extracting the antibiotic from cultures of the transformed agent.

The present invention encompasses the preparation of antifungal formulations in which one or more transformed bacterial strains is used as an active ingredient. The present invention further encompasses the preparation of antifungal formulations in which the active ingredient is Phl produced by the transformed bacterial agent of the present invention. Formulations in which the active ingredient is a bacterial biocontrol agent or the antibiotic Phl produced by such agent may be applied in any manner known for seed and soil treatment with such preparations.

Other uses

To identify *phl* genes from other bacterial species, preferably species of the genus *Pseudomonas*, genomic DNA from a culture of the bacteria is isolated as described below. The isolated DNA is cut with one or more restriction enzymes, cloned in an appropriate vector such as a cosmid vector to generate a library, and DNA from colonies containing clones from the library is blotted onto a nylon membrane such as Nytran. The blots are probed with an oligonucleotide probe containing a *phl* gene or portion thereof greater than 9 nucleic acids in length, and preferably greater than about 18 nucleic acids in length. Probes to specific structural features of the Phl proteins are preferred as they provide a means to isolate *phl* genes with similar structural domains. Most preferred are probes including the catalytic and signature domains of *phlD*, which is required for production of

Phl. Probes may be based on the sequence of either strand of the DNA comprising the motif, and can be degenerate (i.e., a mixture of all possible coding sequences for a given amino acid sequence). General methods for designing and preparing such probes are provided, for example, in Ausubel et al., supra, and *Guide to Molecular Cloning Techniques*, 1987, S. L. Berger and A. R. Kimmel, eds., Academic Press, New York. These oligonucleotides are useful for *phl* gene isolation, either through their use as probes capable of hybridizing to *phl* complementary sequences or as primers for various polymerase chain reaction cloning strategies.

Hybridization procedures and techniques are well known to those skilled in the art and are described in Ausubel, supra, and in *Guide to Molecular Cloning Techniques*, 1987, S. L. Berger and A. R. Kimmel, eds., Academic Press, New York. If desired, a mixture of different oligonucleotide probes may be used for the screening of the recombinant library. Conditions of high stringency for detecting a *phl* gene include hybridization at about 42°C and about 50% formamide; and sequential washes at 65°C in 2X SSC-1% SDS and 0.1 X SSC. Lower stringency conditions can be achieved by one or more of the following: hybridization at 42°C without formamide, washing at 42°C, and washing in 6X SSC-1% SDS followed by 6X SSC-0.1% SDS.

Oligonucleotides derived from *phl* gene sequences can also be used as primers in PCR cloning strategies. PCR methods are well known in the art and described, for example, in *PCR Technology*, H. A. Erlich, ed., Stockton Press, London, 1989; *PCR Protocols: A Guide to Methods and Applications*, M. A. Innes, D. H. Gelfand, J. J. Snitsky, and T. J. White, eds. (1990), and Ausubel et al., supra. *P. fluorescens* contains one genomic region encoding Phl proteins. The conserved regions in the *phl* genes are useful in the design of primers to mediate the recovery of functional *phl* homolog genes in other bacteria, particularly members of the genus *Pseudomonas*. Such primers are designed to amplify only sequences flanked by the oligonucleotides in genes having sequence identity to *phl* genes.

Once a clone encoding a putative *phl* gene is identified, it is then determined whether the candidate gene is capable of producing a functional Phl homolog protein. A functional Phl homolog protein is one encoded by DNA from a source other than *P.*

fluorescens Q2-87 which can, when substituted for the homologous *phl* gene protein from strain Q2-87, function in place of that Q2-87 *phl* gene protein. Assays based on the functional substitution of one protein for another are termed complementation assays and, in bacteria, generally are conducted in a strain in which the gene to be complemented is inactivated by mutation, and the gene to be tested for complementation activity is introduced on a plasmid. Thus, in Table 1 the mutant Q2-87::Tn5-1 was complemented with the plasmid pMON5122, and in Example 7, Q2-87::Tn5-1 was not complemented with a *Bam*HI-*Cla*I fragment from *phlD*.

The availability of the *P. fluorescens* nucleic acid molecules encoding *phl* gene proteins makes accessible *phl* gene sequences encoding *phl* gene proteins or functional homologs from other bacterial strains. The *phl* gene sequences or portions thereof are used as oligonucleotide probes to hybridize to the additional genomic sequences by hybridization under standard conditions. Sequences which hybridize specifically to a *phl* gene coding sequence or its complement and which encode a Phl functional homolog protein are encompassed by the invention. Such oligonucleotides are prepared by standard methods and assembled by procedures known to those in the art. The length of the probe employed must be sufficient to hybridize to homologous regions of DNA wherein hybridization is due to at least about 70% homology, as opposed to nonspecific binding.

It is well known in the biological arts that certain amino acid substitutions can be made in protein sequences without affecting the function of the protein. Generally, conservative amino acid substitutions or substitutions of similar amino acids are tolerated without affecting protein function. Similar amino acids can be those that are similar in size and/or charge properties, for example, aspartate and glutamate and isoleucine and valine are both pairs of similar amino acids. Similarity between amino acid pairs has been assessed in the art in a number of ways. For example, Dayhoff et al. (1978) in *Atlas of Protein Sequence and Structure*, Vol. 5, Supplement 3, Chapter 22, pages 345-352, which is incorporated by reference herein, provides frequency tables for amino acid substitutions which can be employed as a measure of amino acid similarity. Dayhoff's frequency tables are based on comparisons of amino acid sequences

for proteins having the same function from a variety of evolutionarily different sources.

The amino acid sequence of the protein may or may not be identical with the amino acid sequence which occurs naturally in bacteria. The identity of *phl* genes can be confirmed by their ability to produce a functional Phl homolog protein as defined above. In addition, artificially induced mutations can be included so long as they do not destroy activity. A "mutated Phl protein" refers to protein which has the same activity as its unmutated predecessor, but which is derived by mutation of a DNA encoding a Phl protein. By "derived from mutation" is meant both direct physical derivation from a DNA encoding the starting material Phl gene protein using, for example, site specific mutagenesis or indirect derivation by synthesis of DNA having a sequence related to, but deliberately different from, that of the Phl gene. As means for constructing oligonucleotides of the required length are available, such DNAs can be constructed wholly or partially from their individual constituent nucleotides.

Genetically engineered means that the result has been directed by the hand of man. A bacterium genetically engineered to contain a particular DNA molecule is one into which the DNA has been introduced by any means known to the art, including but not limited to transformation, conjugation, electroporation, particle bombardment, and the like. A genetically engineered nucleic acid molecule, such as a genetically engineered DNA molecule, is one which is the product of molecular biological process including but not limited to DNA ligation, *in vitro* mutagenesis or the like.

The DNA sequences of the invention are useful to prepare DNA expression molecules by cloning the sequence in any suitable expression vector that is capable of introducing a foreign gene into a heterologous bacterial host. The recombinant vector is constructed so that the coding sequence is located in the vector with the appropriate control sequence and operationally associated therewith, that is, the positioning and orientation of the *phl* gene DNA coding sequence with respect to the control sequences is such that the coding sequence is transcribed under the control of the control sequences (i.e., by RNA polymerase which attaches to the DNA molecule at the control sequences). The control sequences may be ligated to the coding sequence prior to insertion into a

vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequence and an appropriate restriction site downstream from the control sequence. The vector should be selected so as to have a promoter operable in the host cell into which the vector is to be inserted (that is, the promoter should be recognized by the RNA polymerase of the host cell). In addition, the vector should have a region which codes for a ribosome binding site positioned between the promoter and the site at which the DNA sequence is inserted so as to be operatively associated with the *phl* gene coding sequence once inserted. The vector should be selected to provide a region which codes for a ribosomal binding site recognized by the ribosomes of the host cell into which the vector is to be inserted.

EXAMPLES

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention as claimed. The examples use many techniques well known and accessible to those skilled in the arts of molecular biology and in the manipulation of recombinant DNA in bacteria. Enzymes are obtained from commercial sources and are used according to the vendors' recommendations or other variations known to the art. Reagents, buffers and culture conditions are also known to the art. References providing standard molecular biological procedures include Sambrook et al. (1989) *Molecular Cloning*, second edition, Cold Spring Harbor Laboratory, Plainview, NY; R. Wu (ed.) (1993) *Methods in Enzymology* 218: Wu et al. (eds.) *Methods in Enzymology* 100, 101; Glover (ed.) (1985); *DNA Cloning*, Vols. I and II, IRL Press, Oxford, UK; and Hames and Higgins (eds.) (1985) *Nucleic Acid Hybridization*, IRL Press, Oxford, UK. Abbreviations and nomenclature, where employed, are deemed standard in the field and are commonly used in professional journal such as those cited herein. All references cited in the present application are expressly incorporated by reference herein.

Example 1. Identification of a fragment capable of conferring and enhancing *Phl* production.

A. The genetic region that carries all the information necessary to direct Phl biosynthesis is identified by cloning DNA segments from a Phl-producing strain such as Q2-87 into a suitable broad host range vector such as pRK415 (carries a tetracycline resistance marker) that can be maintained in transformed *Pseudomonas* strains, and introducing the cloned DNA segments into a rifampicin-resistant mutant of the Phl-nonproducer *P. fluorescens* M4-80R via a triparental mating (Thomashow and Weller, *J. Bacteriol.* 170:3499-3508 (1988)). The transformed derivatives were then screened for (1) the presence of a soluble red pigment, the presence of which is correlated with production of Phl; (2) the ability to inhibit in vitro the growth of the indicator bacterium *Clavibacter michiganensis* subsp. *michiganensis*; and (3) production of Phl as determined directly by extraction and analysis by thin layer chromatography.

For triparental matings, overnight cultures (in 5 ml LB with appropriate antibiotic selection) of the donor *Escherichia coli* strain DH5 α carrying plasmids with inserted Q2-87 DNA, and the helper *E. coli* strain HB101 (pRK2013) (provides functions required for DNA transfer by bacterial conjugation) were centrifuged and resuspended in 500 μ l of fresh LB broth. 25 μ l of each of these cultures was spotted onto Millipore filter papers (1cm²) and mixed; after incubation for 2 hours at 37° C, 30 μ l of the recipient strain *P. fluorescens* M4-80R, (10ml LB culture grown overnight at 28° C, centrifuged, and resuspended as above) was added. Filters were incubated overnight at 28° C and the resulting cell mass was resuspended in 1 ml of sterile distilled water. Aliquots of 200 μ l of this suspension were spread on LB plates containing rifampicin and tetracycline.

Rifampicin and tetracycline resistant transformants obtained from triparental matings were evaluated for production of red pigment visually after growth on Yeast Malt (YM) broth or King's Medium B (King, et. al., *J. Lab. Clin. Med.* 44:301-307 (1954)), after incubation at 28°C for 4 to 7 days.

A spontaneous, tetracycline-resistant mutant of *Clavibacter michiganensis* subsp. *michiganensis* was used to assess inhibition in vitro. Inhibition of *C. m. michiganensis* by Phl was analyzed as follows: 100 μ l of a 48-hour old-culture of the indicator organism in water agar (3ml)

was overlayed on YM plates with appropriate antibiotic. These plates were incubated for 4 to 6 h to let the agar solidify and allow initial growth of *C. m. michiganensis*. Strains to be tested for Phl production were spotted onto these plates using a toothpick. Strains were assayed in sets of three per plate and each set was analyzed in triplicate. Each plate also included the positive control strain M4-80R (pMON5122) and the negative control strain, M4-80R (pRK415). The diameter of the zones of inhibition was measured after two to four days and the values were compared statistically among sets by an analysis of variance (ANOVA); the set means were compared by a least significant different test (LSD) when significant differences among sets was indicated by the ANOVA (Fisher's protected LSD).

For extraction of Phl and monoacetylphloroglucinol (MAPG), a possible Phl precursor, cultures were grown for 4 to 7 days either in liquid YM medium or on YM plates. The cells were removed by centrifugation and the antibiotic was extracted from the cell pellet as described by Keel et al. (1992) except that the final extract was dissolved in 100% methanol. The samples were chromatographed on Uniplate™ Silica Gel GHF TLC plates (Alltech Associates Inc. Deerfield, Il) in a solvent system of chloroform:acetone 9:1 (v/v). Spots were visualized by UV absorption at 254nm.

For example, the plasmid pMON5118 and its deletion derivatives, described by Vincent et al. (Appl. Environ. Microbiol. 57:2928-2934 (1991), were introduced into *P. fluorescens* strain M4-80R by triparental mating.

Strain M4-80R carrying any of the plasmids pMON5116, pMON5117, pMON5118, pMON5120 and pMON5123 did not produce the red pigment and did not inhibit *C. m. michiganensis*. Neither Phl nor MAPG could be isolated from any of these strains. On the other hand, strain M4-80R (pMON5122) showed red pigment production, inhibited *C. m. michiganensis*, and both Phl and MAPG were isolated from broth as well as agar cultures of this strain (Table 1). Thus, only plasmid pMON5122 was able to transfer Phl biosynthetic capability to strain M4-80R. The fact that larger fragments (pMON5120, pMON5118 and pMON5117) carrying DNA sequences flanking the fragment in pMON5122 were unable to transfer Phl synthesis may indicate that they carry negative regulator sequences.

Table 1

Plasmids	Q2-87::Tn5			M480R		
	Phl	Red pigment	HCN	Phl	Red pigment	HCN
pMON5118	+	4	5	-	0	0
pMON5117	+	3	5	-	0	0
pMON5120	+	3	5	-	0	0
pMON5122	+	4	5	+	4	0
pMON5123	+	N.D.	5	-	0	0

B. To determine whether the fragment from pMON5122 was able to confer or enhance Phl biosynthetic capability in a wide range of *P. fluorescens* strains, it was cloned as a *Hind*III-*Eco*RI fragment into pVSP41, a plasmid that can be maintained in *Pseudomonas* strains without antibiotic selection. This construction, called pPHL5122, was introduced into 6 *Pseudomonas* strains by triparental matings as described above, and the production of Phl by the transformed strains was assessed by production of red pigment and extraction of Phl from broth cultures as described above. Plasmid pPHL5122 directed Phl synthesis in all of the six transformed recipients. Strains that did not already produce Phl acquired ability to produce the antibiotic. Strains that already produced Phl, i.e., Q2-87, showed an increase in antibiotic production (e.g. Q2-87(pPHL5122), Table 2). pPHL5122 also transferred Phl biosynthetic capability to seven other Phl nonproducing recipient strains into which it was introduced as indicated by the presence of the red pigment. Thus, pPHL5122 conferred or enhanced Phl biosynthesis in all strains into which it was introduced. To determine whether the locus contained on pMON5122 specifically directed Phl production, or if it also affected the production of other secondary metabolites as would be expected of a global regulatory gene, HCN production was assayed in Q2-87, Q2-87::Tn5 (a mutant described by Vincent et al. with a Tn5 transposon that inactivates the genomic homologue of the genes cloned in pPHL5122), and the seven transconjugant derivative strains into which pPHL5122 (or pMON5122) was introduced. Strains were grown on modified KMB medium containing 4.4g/L glycine (Pierson and Thomashow, *Mol. Plant-Microbe Interact.* 5:330-339 (1992)). HCN indicator paper was taped to the lid of the plates and the plates were sealed with Parafilm and incubated for 2 to 4 days at 28° C. HCN indicator paper was prepared by soaking Whatmann 3MM filter paper in a solution of 2% sodium carbonate and 0.5% picric acid and air drying. HCN production was detected by the yellow to red color change of the indicator paper. *P. fluorescens* strain CHA0, which produces copious amounts of HCN, was used as positive control and strain M4R-80, which does not produce HCN, was included as a negative control. Four of the recipient strains (M4-80R, M7z-80, Q26a-80,

and Q29z-80) did not produce HCN, whereas three others (M23dz-80, Q69c-80R2, and Q2-87) were HCN⁺ (Table 2). Both Q2-87 and Q2-87::Tn5 produced similar amounts of HCN as detected by the conversion of the yellow HCN indicator paper to red color, indicating that the Tn5 insertion specifically inactivated production of Phl. Further, HCN production was not coordinately transferred with Phl biosynthesis in the HCN-nonproducing strains upon introduction of pPHL5122, nor was the amount of HCN produced by strain M23dz-80 detectably changed by plasmid pPHL5122. However, HCN production was slightly reduced in Q69c-80 (pVSP 41) and Q69c-80 (pPHL5122) and markedly lessened in Q2-87 (pPHL5122) (Table 2). As expected, pPHL5122 also conferred the Red phenotype characteristic of Phl biosynthesis; otherwise, the appearance of the transgenic strains was unchanged. These results indicate that the fragment contained on pPHL5122 does not mediate global activation of genes involved in secondary metabolism.

Table 2

Strains	Phl synthesis $\mu\text{g/ml}$ culture	Red pigment production	HCN production
M7z-80	0	0	0
M7z-80 (pVSP41)	0	0	0
M7z-80 (pPHL5122)	0.25	2	0
Q26a-80	0	0	0
Q26a-80 (pVSP41)	0	0	0
Q26a-80 (pPHL5122)	600-2000	4	0
Q29z-80	0	0	0
Q29z-80 (pVSP41)	0	0	0
Q29z-80 (pPHL5122)	20-35	4	0
M23dz-80	0	0	1
M23dz-80 (pVSP41)	0	0	1
M23dz-80 (pPHL5122)	10-20	5	1
Q69c-80R2	0	0	4
Q69c-80R2 (pVSP41)	0	0	3
Q69c-80R2 (pPHL5122)	1.0-3.75	4	3
Q2-87	0.5	5	5
Q2-87 (pVSP41)	0	5	5
Q2-87 (pPHL5122)	30-200	5	2

C. We have used the mini-transposon vector pUT mini-Tn5 Km described by de Lorenzo, et al., supra, to introduce *Phl* biosynthetic genes from pMON5122 into the chromosomes of rhizosphere pseudomonads including strains Q69c-80, Q65c-80, and the PCA-producing strains 2-79 and 30-84. The *Hind*III-*Eco*RI fragment from pMON5122 was cloned into the plasmid vector pUCNotI such that *Not*I restriction sites were added at either end of the fragment, and the fragment was then excised by digestion with *Not*I and ligated into the *Not*I site in the vector pUT::Km. This site is located between two IS-terminal sequences that also flank a transposable miniTn5 element carrying a kanamycin resistance gene, and pUT::Km also carries a transposase gene adjacent to but outside of the mobile DNA segment (Herrero et al, *J. Bacteriol.* 172:6557-6567 (1990)). The resulting plasmid, pUT::Km-*Phl*, can only replicate in bacterial strains that provide an R6K-specified π protein not produced by recipient strains of *Pseudomonas*.

Transfer of the plasmid pUT::Km-*Phl* from the donor strain *E. coli* S17-1 λ pir, into the *Pseudomonas* recipient strains Q69c-80, Q65c-80, 2-79 and 30-84 was by conjugation. The donor strain, which provides the necessary conjugation factors, was grown overnight with shaking at 37° C in 5 ml of LB broth amended with ampicillin and kanamycin (each 100 μ g/ml). Each rifampicin-resistant recipient pseudomonad was grown overnight in 5 ml of LB broth without antibiotics. The cultures were centrifuged at 6,000 rpm for 5 min and resuspended in 1 ml of fresh LB broth. For each recipient strain, thirty μ l of the donor was spotted onto a membrane filter placed on an LB plate, and then 20 μ l of cell suspension of one recipient strain was added and the two were mixed. These plates were incubated at 27° C overnight, and then the bacterial growth on each filter was suspended in 1 ml of sterile distilled water, centrifuged, and resuspended in 1 ml of sterile distilled water. The washed cells were diluted 10-fold, and 100 μ l of each dilution was plated on M9 minimal medium (0.3 g KH_2PO_4 , 0.5 g sodium chloride, 1.0 g ammonium chloride, 1 ml calcium chloride (50 mM), 1 ml $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 M)) amended with 100 μ g/ml of kanamycin ($\text{M9}_{\text{kan100}}$) and incubated at 27° C for 3 days. By selecting for kanamycin-resistant transconjugants from this mating, strains were obtained in which the mini-Tn5 element containing *phl* genes and the kanamycin resistance gene had

transposed into the *Pseudomonas* genome.

Twenty kanamycin-resistant transposition derivatives of Q69c-80 have been evaluated for the presence of *phl* genes and for Phl production. Strain Q69c-80::mTn5PHL20 was deposited under terms of the Budapest Treaty June 21, 1995, in the Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois, and has been assigned accession number NRRL B-21474. Southern blot analysis (Photogene Nucleic Acid Detection System, BRL®, Life Technologies, Inc., Gaithersburg, MD 20898, USA) was carried out to confirm insertion of the Phl biosynthetic locus and reveal the insertion pattern of the locus on the chromosome of *P. fluorescens* Q69c-80. For Southern hybridization, genomic DNA was prepared as described by Ausubel et al., supra. Cultures were grown overnight in 1.5 ml of LB broth, the bacterial cells were centrifuged and resuspended in a lysis solution (561 μ l $T_{10}E_1$ buffer, 15 μ l of 20% SDS, and 12 μ l of 5 mg/ml Pronase®) and incubated at 37°C for 1 h. One hundred two microliters of 5 M NaCl was added and mixed thoroughly. Eighty microliters of CTAB/NaCl solution was added and mixed, and the suspension was incubated at 65°C for 10 min. The DNA was extracted sequentially with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and with chloroform /isoamyl alcohol (24:1). The DNA was precipitated with 60% volume of isopropanol and washed with 70% ethanol before resuspending in $T_{10}E_1$ buffer.

Genomic DNA from each of the 20 derivatives was digested with *Sal*I (first experiment) or *Eco*RV (second experiment). Digested DNA was separated by electrophoresis on a 0.7% agarose gel, and transferred and bound to a nylon membrane (Magnagraph®, Micron Separations Inc., Westboro, MA 01581, USA) by known methods.

Eight hundred microliters (5 mg/ml) of salmon sperm DNA (Sigma, St. Louis, MO 63178, USA) was boiled for 10 min and diluted into 40 ml of hybridization solution (1x HPB, 1% SDS). The membrane and the hybridization solution were introduced into a sealable plastic bag and incubated for 2 to 4 h at 65°C.

A DNA probe consisting of the excised and purified fragment from pMON5122 labeled as directed by the manufacturer with the BioNick Labeling System (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD 20898, USA) was boiled for 10 min, added to the plastic bag, and incubated overnight at 65°C. After hybridization,

the membrane was washed twice in 100 ml of a solution (5x SSC, 0.5% SDS) at 65° C for 5 min per wash, once with 100 ml of a solution (0.1x SSC, 1% SDS) at 65° C for 30 min, once in 100 ml of 2x SSC at room temperature for 5 min, and then incubated in 100 ml of blocking solution (Tris-buffered saline (TBS)-Tween 20, 3% BSA) for 1 h at 65° C. Seven microliters of streptavidin-alkaline phosphatase (SA-AP) was diluted into 7 ml of TBS-Tween 20 (1:1000 dilution), and the resultant solution was poured on the membrane and incubated at room temperature for 10 min with repeated pipetting. The membrane was washed twice in 100 ml of TBS-Tween 20 at room temperature for 15 min each time and in 100 ml of the BRL final wash buffer at room temperature for 1 h. The membrane was briefly blotted to remove excess liquid and placed on a plastic development sheet. One milliliter of the development reagent was dropped near the membrane on the plastic development sheet and immediately covered with another plastic sheet to spread the reagent evenly under conditions of reduced light. After 30 min, a piece of film was placed over the membrane, exposed for 2-4 h and developed. Results from Southern hybridization indicated that single copies of the Phl genes had transposed to unique sites in the individual derivatives.

All of the derivatives initially produced Phl in vitro as indicated by the presence of the soluble red pigment described above. Phl production was quantified by using High Pressure Liquid Chromatography (HPLC). Pseudomonads were grown with shaking (200 rpm) in 15 ml culture tubes with 4.0 ml yeast malt broth for 60 h at room temperature. Cultures were acidified with 125 μ l of 3.0 N HCl, and extracted twice with 10 ml ethyl acetate (90% extraction efficiency). The organic phase contained Phl and MAPG and was evaporated to dryness and suspended in 3.0 ml of 35% (v/v) of acetonitrile (ACN);water both containing 0.1% trifluoroacetic acid (TFA). Crude extracts were filtered through 0.20 μ m syringe filters, followed by C-18 reverse phase HPLC (Waters NOVA-PAK C-18 Radial-PAK cartridge, 4 μ m, 8 x 100 mm). The Waters HPLC system consisted of a 710B WISP, 510 pumps, and 680 automated gradient controller, with a 990 photo-diode array detector. HPLC solvent conditions were at a flow rate of 1.0 ml/min, with a 2 min initial condition at 10% ACN/water/0.1%TFA, followed by a 20 min linear gradient to 100% ACN/0.1% TFA. HPLC

gradient profiles were monitored at the spectral peak maxima characteristic for Phl in the designated solvent system (270 nm, 330 nm). Phl eluted at a retention time of 17.02 min at approximately 78% ACN/0.1% TFA. Standard curves were run using complementary Phl-nonproducing strains, where cultures spiked with known concentrations of standard were subjected to the same extraction procedure described above. Seven point, extracted, standard curves were completely linear and reproducible yielding correlation coefficients ranging from 0.9997-0.9994.

Table 3

Derivative	A ₆₀₀	µg/ml of culture	µg/g dry wt of culture	µg/A ₆₀₀
Q69C-80 (pPHL5122)	--	8	0.33	--
Q69C-80 (pVSP41)	--	n.d.	--	--
Q69C-80::mTN5 Ph1 1	1.06	87	3.3	329
Q69C-80::mTN5 Ph1 2	1.09	59	2.6	213
Q69C-80::mTN5 Ph1 3	0.92	153	6.5	665
Q69C-80::mTN5 Ph1 4	1.96	0.4	0.03	0.7
Q69C-80::mTN5 Ph1 5	1.26	24	1.0	76
Q69C-80::mTN5 Ph1 6	1.21	34	1.5	111
Q69C-80::mTN5 Ph1 7	0.70	153	6.7	870
Q69C-80::mTN5 Ph1 8	1.10	40	1.7	146
Q69C-80::mTN5 Ph1 9	0.76	196	8.1	1,034
Q69C-80::mTN5 Ph1 10	0.83	143	6.3	690
Q69C-80::mTN5 Ph1 11	0.82	173	7.0	850
Q69C-80::mTN5 Ph1 12	1.05	32	1.3	121
Q69C-80::mTN5 Ph1 13	0.87	175	7.4	806
Q69C-80::mTN5 Ph1 14	0.76	91	4.0	483
Q69C-80::mTN5 Ph1 15	0.95	85	4.0	409
Q69C-80::mTN5 Ph1 16	0.98	87	4.0	352
Q69C-80::mTN5 Ph1 17	0.81	104	4.8	540
Q69C-80::mTN5 Ph1 18	0.71	149	6.4	832
Q69C-80::mTN5 Ph1 19	0.99	95	4.0	383
Q69C-80::mTN5 Ph1 20	0.83	97	4.5	465

Considerable variation was observed among individual strains in growth on yeast-malt broth, stability of the phenotype (there was a strong tendency to loss of red pigmentation and failure to produce detectable Phl), and amount of Phl produced (Table 3). Quantities of Phl sometimes were over twenty-fold greater than produced by Q69c-80(pPHL5122), and Phl usually was not detectable in cultures of strain Q2-87 grown under comparable conditions (data not shown). These results suggest that expression of the transposed genes is deregulated, a hypothesis supported by DNA sequence data indicating that *phlF*, a putative repressor of Phl synthesis, is truncated and probably unable to repress *phl* gene expression in constructs containing the sequences shown in SEQ ID NO:4.

Example 2. Generation and characterization of mutants to delineate the size of the fragment capable of conferring Phl biosynthetic capability.

A restriction map of the DNA fragment cloned into pMON5122 was generated using the following restriction enzymes: *Bam*HI, *Bst*EII, *Cla*I, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Pvu*II, and *Sal*I. This map revealed the cloned fragment to be 6.5 kb in size, and thus substantially smaller than the 9.5 kb value that was reported by Vincent et. al., supra. The relative positions of restriction sites were, however, sufficiently similar to those reported by Vincent et al. to suggest that Vincent's size estimate was in error, rather than that the maps were of two entirely different DNA segments.

That portion of the cloned fragment that is necessary to direct Phl biosynthesis was determined by mutating pMON5122 with the transposon Tn3HoHo1 (Stachel et. al., *The EMBO J.* 4:891-898 (1985)). Insertions of Tn3HoHo1 were obtained by transforming *E. coli* strain HB101(pHoHo/pSShe) with pMON5122. Transformants containing all three plasmids were subsequently mated with *E. coli* containing the conjugal helper plasmid pRK2013 and *E. coli* C2110, a nalidixic acid-resistant, *polA*-deficient recipient strain which does not support replication of pHoHo and pSShe. Donor, helper and recipient strains were spotted onto Millipore filters (1cm²) and incubated at 37° C for 6 hours. The growth on the filters was suspended in 1 ml of sterile distilled water and 200 µl of this

suspension was spread on LB agar containing tetracycline (to select for pMON5122), ampicillin (to select for those plasmids containing the transposed Tn3HoHo1 element) and nalidixic acid (selects strain C2110). Transconjugants resistant to all three antibiotics were isolated in 18 sets of 48 on a grid. The site and orientation of insertion of the transposon in each pMON5122 derivative was analyzed by digestion of isolated plasmid DNA with *EcoRI*, and in triple digests with *HindIII*, *BamHI* and *EcoRI*. Of 846 mutants that were analyzed, 19% contained transposon insertions within the cloned fragment. Duplicate insertions giving rise to identical restriction fragments were obtained with high frequency in the left half of the fragment, whereas few insertions were obtained within the right end. In all, 43 unique insertions were analyzed further.

Those plasmids containing insertions within the cloned fragment then were introduced by triparental mating into M4-80R and tested for the ability to direct Phl biosynthesis by TLC and by the *C. michiganensis* subsp. *michiganensis* inhibition bioassay described in Example 1. The bioassay method (detection limit 250 ng) was more sensitive than TLC (detection limit about 1 µg) and therefore was chosen for evaluation of the effect of the various insertions on Phl synthesis by strain M4-80R carrying the mutated plasmids.

In Table 4, the value given for each insertion represents the percent decrease or increase in the size of the zone of inhibition on bioassay plates, relative to the positive control strain M4-80R(pMON5122) within one assay set. An assay set consisted of positive and negative controls and three different insertion derivatives of M4-80R(pMON5122). For each insertion, statistical comparisons were made only within assay sets by analysis of variance and Fisher's protected least significant difference test at $p=0.05$. ** indicates not significantly different from the positive control within the same assay set.

* indicates % reduction of inhibition was intermediate between the positive and negative control ($p=0.05$) and the phenotype was Red⁻. M represents the average value for the negative control across all assay sets.

Table 4

Insertion	% Reduction	Insertion	% Reduction
2	67	23	36
3	54	24	29
4	57	25	28*
5	74	26	17
6	66	27	24
7	55	28	8**
8	13**	29	23
9	19**	30	-7**
10	74	31	20**
11	46	32	32**
12	57	33	31*
13	68	34	22
14	56	35	24
15	70	36	32
16	67	37	22
17	66	38	34
18	31	39	27
19	46	40	56
20	56*	41	76
21	36**	42	80
22	32	43	68
		M	72

M4-80R containing derivatives of pMON5122 with insertions at positions 2 through 7 and 10 through 18, located within a 5-kb region at the right end of the fragment shown in Figure 2, were significantly less inhibitory of *C. m. michiganensis* than was M4-80R(pMON5122), indicating partial or full loss of Phl production (Table 4). Insertion derivatives 8 and 9, which map to approximately the same site but contain Tn3HoHol in opposite orientations, enabled levels of *C. m. michiganensis* inhibition that were not significantly different from those of M4-80R(pMON5122), the positive control. Insertion derivative 8 was, however, Lac⁺ indicating no break in transcription. Insertion derivatives 34 through 39 at the left end of the fragment shown in Fig. 2 were not significantly less inhibitory than the positive control. Thus, only the sequences within the 5-kb region at the right end of the fragment were required for inhibition of the indicator organism and for production of Phl.

Strain M4-80R containing the various pMON5122::Tn3 HoHol derivatives also was evaluated visually for the presence of the red pigment after 4-7 days in YM broth or on KMB agar plates. Whereas all insertions up to and including number 18 that were Phl⁻ were also Red⁻, insertions downstream of number 18 exhibited other phenotypes. Most of these insertions had no effect on the presence of the red indicator pigment. However, insertion derivatives 20, 25, and 33 were Red⁻ and had a level of inhibition of *C. m. michiganensis* intermediate between the positive and negative controls (Figure 2, Table 4). The region encompassing these insertions was therefore designated "Red" in Figures 1, 2, and 3. While not required for Phl production, it clearly is involved in Phl production or activity.

Example 3. Determination of size, orientation and number of potential transcriptional units within the fragment required for Phl biosynthesis.

The M4-80R(pMON5122::Tn3HoHol) derivatives described in Example 2 were evaluated for expression of the *lacZ* gene carried on the transposon Tn3HoHol to determine the presence, number, size, and orientation of the transcriptional units within the mutated sequences. Expression of the *lacZ* gene in each derivative was detected by the development of blue colored colonies on LB

medium containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal). Results of this analysis are indicated in Figure 2. In strain M4-80R, all insertions which resulted in expression of *lacZ*, except three, were oriented in the same direction; only the Lac⁺ insertions 2 and 3 at the right end and insertion 38 at the left end of the locus were oriented in the opposite direction. These results indicate the presence of at least two divergent transcriptional units. The long unit running from right to left continues past the region strictly necessary for the biosynthesis of Phl and into the Red region. The smaller unit running from left to right also extends beyond the fragment present in pMON5122.

Reporter derivative strains of Q2-87::Tn3HoHol-15 and Q2-87::Tn3HoHol-40 were generated by introducing two of the Tn3HoHol insertions (15 and 40) into the Q2-87 chromosome by a process called marker exchange, which occurs due to spontaneous recombination between the sequences on the plasmid flanking the Tn3HoHol insertion and homologous sequences in the chromosome. In practice, such process is directed by the hand of man and the desired result is identified by man.

Strain M4-80R carrying pMON5122::Tn3HoHol derivatives 15 and 40 were inoculated into LB broth without tetracycline; after incubation overnight at room temperature, 100 μ l of the culture was transferred to fresh LB broth. After a second such transfer, serial dilutions of the culture were prepared and 200 μ l of each was plated on LB agar containing (X-gal). Cells in which homologous recombination had taken place gave rise to blue colonies, and could no longer grow on LB agar containing tetracycline. The reporter *lacZ* gene introduced into Phl locus in the chromosome of Q2-87 provides a simple means of monitoring Phl gene expression either visually, as colony color on LB agar containing X-gal, or quantitatively, with a β -galactosidase assay readily accessible to those in the art.

Example 4. Analysis of the region including the Phl biosynthetic genes.

Figure 1 depicts the genetic organization of the 7-kb region which encompasses the entire region known to be involved in Phl production. A variety of subclones were prepared as double-

stranded templates for automated sequencing by digestion of the 6.5-kb fragment from pMON5122 with various restriction endonucleases, either singly or in combination, followed by ligation with the cloning vector pIC19H (Marsh, et. al., Gene 32:481-485 (1984)) (Figure 3). A fragment of DNA which overlaps the right end of the pMON5122 fragment also was cloned from pMON5120 to determine the end point of *phlF* (Figure 3).

Sequences were determined at the Nucleic Acid Research Facility at Iowa State University with an ABI automated sequencer. Sequence analysis was performed with the University of Wisconsin Genetics Computer Group (GCG) software (Staden, et. al., Program Manual for the GCG package, Version 7.2 (Incremental Release - Documentation Updates), October 1992, 575 Science Drive, Madison, Wisconsin, USA 53711 (1992)). The program MAP was used to predict potential open reading frames in the sequence which was further supported by the results of the program CODONPREFERENCE using the *Pseudomonas* codon preference table. The program TRANSLATE was used to translate the open reading frames into protein sequences. The coordinates of the open reading frames in SEQ ID NO:3 are as follows:

<i>phlA</i>	4285-5076;	transcribed from right to left
<i>phlB</i>	2606-3313;	transcribed from right to left
<i>phlC</i>	3085-4251;	transcribed from right to left
<i>phlD</i>	1398-2444;	transcribed from right to left
<i>phlE</i>	2-1270;	transcribed from right to left
<i>phlR</i>	3514-4767;	transcribed from left to right
<i>phlF</i>	5781-6386;	transcribed from left to right

The coordinates of the truncated *phlF* open reading frame in SEQ ID NO:4 are as follows:

phlF (truncated) 5781-6170; transcribed from left to right

Protein sequences were compared to known proteins from all organisms within the NCBI database using the program BLAST (Altschul et. al., *J. Mol. Biol.* 215:403-410 (1990)). The predicted proteins were analyzed for the presence of characteristic sequence motifs using the program MOTIFS and the PROSITE database (Bairoch, *Nucleic Acids Res.* 19:2241-2245 (1991)). This analysis has led so far to the genetic organization of the fragment shown in Figure 1 as well as potential functions for some of the predicted proteins.

The significance of the similarity of a predicted protein to known proteins is determined by calculating the binary comparison score (Z-score), which is measured in standard deviations. To have significant similarity the score must be 3 or greater. A value of 6 or greater corresponds to a probability of about 10^{-9} that the degree of similarity seen in the two sequences arose by chance. Values in this range suggest a common origin by divergent evolution but do not rule out convergent evolution. When a score is greater than 9 the degree of similarity is considered to be too great to have arisen by chance or for the proteins to have evolved convergently so the sequences are considered to be homologous.

phlA predicted PhlA, a protein of 264 amino acids that has no similarity to any proteins in the database but that shows partial conservation (mismatch=1) of a short chain alcohol dehydrogenase family signature, an ornithine/diaminopimelic acid/arginine decarboxylase family signature and a phosphopantetheine attachment site.

Short-chain alcohol dehydrogenase family signature

		*		#									
Consensus		Y(A)	(G)	(A)	K	x(A)	(A)	x{2}	(L)				
PhlA		Y	A	G	A	G	A	A	A	L	L	L	

* - marks the tyrosine residue important for catalytic activity and/or substrate binding

- marks the lysine residue to which the pyridoxal phosphate binds; it is replaced by a glycine residue in PhlA.

Ornithine/Diaminopimelic acid/Arginine decarboxylase family signature

						*	*	*				
Consensus		(S)	x{2}	(F)	(S)	(L)	G	G	G(L)	(G)		
PhlA		S	T	P	F	S	L	G	K	G	L	G

* - marks the three glycine residues proposed to be part of a substrate-binding region.

Phosphopantetheine attachment site

						*						
Consensus		(I)	G(A)	(D)	S(I)	x{3}	(T)	x{4}	(L)			
PhlA		I	G	A	D	T	I	N	RN	T	APGD	L

* - marks the serine to which the phosphopantetheine moiety is attached via its hydroxyl moiety. In PhlA this serine is replaced by threonine which has a similar reactive hydroxyl moiety. Symbols: | - represents identical residue; : - represents conservative replacement; . - represents semi-conservative replacements based on the similarity scores assigned in a PAM120 matrix.

Short chain alcohol dehydrogenases catalyze the reversible oxidation of an alcohol to an aldehyde and are involved in many types of biosynthetic reactions. For example, in bacteria, acetoacetyl- CoA reductase (a member of the short-chain alcohol dehydrogenase family) in *Zooglea ramigera* and *Alcaligenes eutrophus* (Persson, et. al., *Eur. J. Biochem.* 200:537-543(1991)) is involved in the biosynthesis of poly- β -hydroxybutyrate. Ornithine, arginine and diaminopimelic acid decarboxylases are enzymes that act in the biosynthesis of polyamines and lysine, respectively. Phosphopantetheine is the prosthetic groups of acyl carrier proteins (ACP) in some multienzyme complexes where it serves as a "swinging arm" for the attachment of activated fatty and amino acid groups.

phlB predicted protein PhlB, of 236 amino acids. PhlB did not show similarity to any known protein in the database nor did it show conservation of any common protein signatures. PhlB therefore may be a unique, as yet unidentified protein.

phlC predicted PhlC, a protein of 389 amino acids with 27.9% identity and 49.7% similarity to the N-terminal portion of mammalian peroxisomal sterol carrier protein X (SCPx); the Z score for the two is 15; i.e., it is homologous to SCP-X. The 400 N-terminal amino acids of SCPx function as a 3-oxoacyl-CoA thiolase (Seedorf et. al., *J. Biol. Chem.* 269:21277-21283 (1993)); a hypothetical substrate binding site containing a cysteine residue and a glycine-rich C-terminal domain are conserved (Figure 5; Ossendorp et. al., *Eur. J. Biochem.* 201:233-39 (1991)).

Putative substrate binding site:

				*
Rat SCPx	78	SLGLTGIPIINVNNNCSTGST		
		. :.. .: .. :.. .		
PhlC	73	ALGISPAPTFMSTANCTSSSV		

* - marks the reactive cysteine residue.

Glycine-rich C-terminal conserved region

```

Rat SCPx  343 NPSGGLISKGHPLGATGLA
              |..|| |::||: |..|:
PhlC      337 NTDGGNIGRGHASGCDGIL

```

Symbols: | - represents identical residue; : - represents conservative replacement; . - represents semi-conservative replacements based on the similarity scores assigned in a PAM120 matrix.

The thiolase domain of SCPx efficiently catalyzes the thiolytic cleavage of a wide variety of 3-oxoacyl-CoA substrates, with preference for medium-length substrates; it is hypothesized to supply acetyl-CoA for anabolic reactions when cells are well-supplied with energy (Seedorf et. al., *J. Biol. Chem.* 269:21277-21283 (1993)). Thus, PhlC may provide a source of starter units for the synthesis of Phl. Alternatively, thiolases share conserved residues in their condensing domains with β -ketoacyl synthases and chalcone synthases (M. Siggard-Andersen, *Protein Seq. Data Anal.* 3:325-335 (1993)), and the similarity we have detected between PhlC and SCPx may be consistent with activity analogous to chain elongation in fatty acid and polyketide synthesis.

phlD predicted a protein, PhlD, of 349 amino acids with 27.9% identity and 48.0% similarity to members of the chalcone synthase/stilbene synthase (CHS/STS) family of enzymes from higher plants. Randomized binary comparisons of the amino acid sequences of PhlD and CHS from tomato gave a Z-score of 19.5. Thus, PhlD is homologous to chalcone synthases from plants.

PhlD contained regions with extensive similarity to the active site and signature sequence domains of CHS/STS enzymes (Figure 6), but had surprisingly little similarity to prokaryotic β -ketoacyl synthases, including those that catalyze similar condensing reactions (e.g., polyketide synthesis by actinomycetes) in bacteria. It is, to our knowledge, the first prokaryotic protein to show such extensive similarity to the CHS/STS protein family. These observations, and the apparent functional and biochemical parallels between plant defense by Phl-producing plant-associated microorganisms and plant self-defense mediated by phytoalexins (products of CHS/STS enzymes), provoke speculation as to possible gene exchange between plants and their bacterial colonists or more

likely, a common evolutionary origin for these two mechanisms of defense.

Chalcone synthases function as polyketide synthases to catalyze condensation reactions. For example, the CHS from *Lycopersicon esculentum* catalyzes the sequential addition of 3 acetyl units from malonyl-CoA to the phenylpropanoid ring of 4-coumaroyl-CoA and cyclization of the enzyme-bound intermediate to form naringenin chalcone, the central intermediate in the biosynthesis of many flavonoids. CHS accepts aliphatic-CoA esters as starter molecules, and acylphloroglucinols have been recovered as by-products of chalcone synthesis in vitro. This suggests that PhlD may be sufficient to catalyze the condensation and/or cyclization reactions required for MAPG synthesis (Figure 4).

phlE predicted PhlE, a protein of 423 amino acids, that had 24.1% identity and 49.7% similarity to *Staphylococcus aureus* norA protein, a multidrug efflux transporter that contributes to fluoroquinone resistance. The Z score for the two proteins was 10.8. NorA and other such proteins are members of a very large superfamily of transmembrane solute facilitators that extends throughout prokaryotes, cyanobacteria, lower eukaryotes and higher plants and animals. Transporters specific for simple sugars, oligosaccharides, organic acids, organophosphate esters and drugs have been identified; among the most well-known representatives of the drug efflux subfamily are the tetracycline-H⁺ antiporters driven by proton motive force. PhlE retained conserved structural features of these integral membrane permeases (Figure 7), including a central hydrophilic loop bordered on either side by six hydrophobic α -helices.

The region designated Red based on the results of Tn3HoHol mutagenesis (see Example 2) includes phlE. The homology between PhlE and known multidrug efflux proteins suggests that PhlE may function to export Phl from the cell, where it can react with media constituents or oxygen to produce the red pigment. That export occurs under native conditions is indicated by the fact that Phl is recovered in significant quantity from culture media (Table 3). Phloroglucinol (unacetylated) can condense with the coniferaldehyde moiety of lignin compounds from plants to give a reddish color; the reactive group here is the carbonyl of the aldehyde. Such reactive carbonyl groups may be present on

compounds naturally present in media, or may be produced in media by growth of the Phl-producing bacteria.

phlR predicted protein PhlR, of 418 amino acids. PhlR did not show similarity to any known proteins in the database but showed partial conservation (mismatch=1) of the thiolases 3 signature sequence.

Thiolases signature 3

					*							
Consensus	(A)	(I)	x	(G)	x	C	x	G	x	G	x	(G)
PhlA	K	I	F	G	G	C	E	G	K	G	V	G

* - marks the active site cysteine residue involved in deprotonation in the condensation reaction.

Symbols: | - represents identical residue; : - represents conservative replacement; . - represents semi-conservative replacements based on the similarity scores assigned in a PAM120 matrix. Type II thiolases are involved in the thiolysis of acetoacetyl-CoA and in biosynthetic pathways such as poly- β -hydroxybutyrate synthesis and steroid biogenesis.

phlF predicted PhlF, a protein of 202 amino acids with 23% identity and 47% similarity to tetracycline repressor protein. Conservation was concentrated within a helix-turn-helix (HTH) domain typical of known DNA-binding regulatory proteins such as λ cro and repressor proteins, phage 434 cro and repressor proteins, lac repressor, catabolite reactive protein (CRP), and *E. coli* tetracycline repressor protein. The HTH score obtained by using the Helix-Turn-Helix program in the EGCG package (Rice, P., Computer Group, European Molecular Biology Laboratory, Postfach 10-2209, 69012 Heidelberg, Germany) was higher for PhlF than were the scores for λ cro and repressor proteins and the phage 434 cro and repressor proteins. This score is a measure of the probability that the amino acid sequence predicts a functional DNA-binding domain, and strongly supports a role for PhlF in the regulation of gene expression.

It is known that deletions of the Tn10 and class E tetR tetracycline repressor genes resulting in truncation of the carboxy-terminal end of the encoded repressor proteins are derepressed in expression of the tetracycline resistance protein. The very high levels of Phl produced by transformed derivatives of *P. fluorescens* Q69c-80 containing a single chromosomal copy of a

fragment including the truncated *phlF* gene and the *phlACBDE* and *phlR* genes (Table 3) argues that the *phlF* gene is a repressor of *phl* gene expression, and that truncation of *PhlF* permits derepressed synthesis of Phl in a manner analogous to that reported in the tetracycline resistance operon. The fact that pMON5120, containing the intact *phlF* gene, does not transfer Phl biosynthetic capability to the nonproducer strain M4-80R further strengthens this argument.

Subcloning experiments are performed with the objective of determining whether the intact *phlF* gene is sufficient to repress expression of *phl* biosynthetic genes. The *phlF* gene is cloned in its entirety from pMON5120 on an *EcoRV* fragment, *Bam*HI adapters are ligated to the ends, and the fragment is cloned into the *Bam*HI site in pRK415, a broad host-range vector that can be maintained in *Pseudomonas* strains. This construction is introduced into Q2-87::Tn3HoHol-15 and Q2-87::Tn3HoHol-40, reporter strains in which Tn3HoHol transposon insertions 15 (in *phlD*) 40 (in *phlA*) have been introduced into the chromosome of Q2-87 by marker exchange (homologous recombination). The *Bam*HI fragment is sufficient to repress expression of *phlA* and *phlD*, as indicated by loss of blue colony color on media containing the chromogenic substrate X-gal, or by failure to detect β -galactosidase activity in a standard enzyme assay known to those skilled in the art. In contrast, introduction of pRK415 alone causes no change in colony color.

Most of the proteins predicted by each of the *phl* genes showed similarities to proteins with known functions. However, since the pathway for synthesis of Phl in *Pseudomonas* is at present unknown, and because the identified sequence similarities are to proteins other than those known to catalyze polyketide biosynthetic reactions in bacteria (such as actinomycetes), the actual role of the predicted proteins in Phl synthesis is yet to be determined. The cloned genes described herein permit fundamental studies on the mechanism of Phl biosynthesis in *Pseudomonas* spp. by providing information and materials for the generation of clones to express individual *phl* genes and to analyze the activities and roles of their products in the biosynthesis of Phl.

Example 5. The native promoters for *phlF* and for *phlA*, *phlB*, *phlC*, *phlD* and *phlR* are contained within SEQ ID NO:4.

The majority of the Tn3HoHol insertions in M4-

80R(pMON5122::Tn3HoHol) derivative strains that were Lac⁺ were oriented from right to left; only insertions 2, 3 and 38 were oriented from right to left (Figure 2). The *lac* promoter in the vector pRK415 is located distal to the left end of the cloned fragment as shown in Figure 2 and could direct expression of the *lacZ* gene only in M4-80R(pMON5122::Tn3HoHol-38). Expression of the *lacZ* genes in the remaining Tn3HoHol insertions therefore must be driven from promoters other than those in the vector. There were two sets of such insertions, one including those numbered 4, 5, 6, 8, 10, 14, 15, 16, 18, 19, 20, 21, 23, 24, 27, 28, 31, 32, 34, 37, and 39 in Figure 2, and the other including insertions 2 and 3. As these two sets of insertions are divergently oriented, the cloned fragment illustrated in Figure 2 must contain at least two divergently oriented transcriptional units and two promoters situated between *phlA* and *phlF* that are the native promoters of these genes.

That this region carried promoter sequences was supported by the nature of its sequence. There were few or no open reading frames, and the sequence was very high in the bases A and T, which is characteristic of promoter sequences.

Example 6. Cloning of the *phlD* gene and expression of its gene product.

a. Cloning of *phlD*. The *phlD* gene is cloned as follows based on results of sequence analysis: the 5' end of *phlD* is cloned as a *AccI*-*SalI* fragment into pIC19H cut with *ClaI* and *SalI*. The remaining 3' portion of the *phlD* is then introduced into the *SalI* site to regenerate the entire *phlD* gene in plasmid pPHLD. The cloned fragment from pPHLD is isolated as a *HindIII* fragment and cloned into pTrcHis expression vectors A, B, and C (Invitrogen) to express the PhlD protein for assays of enzyme activity. The fragment is also cloned into the broad host range vector pRK415, and the resulting construction is introduced into Q2-87:Tn5-1 by triparental mating to determine if it is sufficient to complement the mutation in the chromosomal *phlD* gene. The transconjugant so generated is tested for Phl biosynthesis by production of red pigment, bioassay, and extraction of Phl and MAPG.

b. Expression of *phlD* and assay of enzymatic activity of expressed protein. For the preparation of active enzyme, the

cells are grown, expression is induced, and the protein is isolated according to manufacturer's instructions. To assay enzyme activity, mix 15 μ l of the isolated enzyme, 75 μ l of buffer (0.2 M Hepes/KOH (pH 7.0), 1mM EDTA), 5 μ l of starter unit (e.g., 4-acetoacetyl-CoA or acetyl-CoA (1nmol)), and 5 μ l of extender unit (e.g., [2-¹⁴C]malonyl-CoA (1.5nmol, 0.78GBq/mmol, Amersham Corp.)). Incubate 30 min at 37°C; stop reaction by two sequential extractions with 0.15 ml of ethyl acetate. The solvent is removed by evaporation under vacuum (Speedvac), and the residue is dissolved in 10 μ l ethyl acetate and analyzed by TLC in 15% acetic acid. Negative control samples spiked with Phl and/or MAPG are used to quantitate the efficiency of recovery (Lanz et al., *J. Biol. Chem.* 266:9971-9976 (1991)). Detection is by scintillation counting of samples scraped from the TLC plate.

Cerulenin binds to the active site cysteine of condensing enzymes and is known to inhibit the activity of chalcone synthases. For cerulenin inhibition assays, cerulenin is dissolved in ethylene glycol monomethyl ether. Reaction mixes (as above) are pre-incubated with this solution for 10 min at 25°C before starting the reaction by adding substrates.

Example 7. Intact *phlD* is necessary for Phl synthesis.

The Tn5 insertion in Q2-87:Tn5-1 is located with *phlD*; this mutation completely eliminates production of Phl as well as MAPG, suggesting that *phlD* is essential for the production of Phl. The *Bam*HI-*Cla*I fragment that encodes the 293 amino acids at the 3' end of the 349 amino acid PhlD protein was cloned into pIC19H, excised as *Hind*III fragment, cloned in both orientations into the broad-host range vector pRK415 and introduced into Q2-87:Tn5-1 and into strain M4-80R by triparental mating. In either orientation the cloned fragment was unable to complement the mutation in Q2-87:Tn5-1 and did not direct Phl synthesis in M4-80R. Thus, intact PhlD is necessary for Phl synthesis.

Example 8. Improvement of biocontrol capability of strains transformed with DNA sequences that confer or enhance Phl production.

a. Preparation of *Gaeumannomyces graminis* var. *tritici* inoculated soil. Isolates of *G. g. tritici* were started from

single ascospores from the roots of diseased wheat plants. The isolates were maintained on fifth-strength potato dextrose agar (PDA) (Duffy and Weller, *Phytopathology* 84:1407-1415 (1994)). To prepare *G. g. tritici* inoculum, an isolate was grown for 5-7 days on PDA in petri plates and then transferred as mycelium and accompanying agar medium to a 1-liter flask containing autoclaved oat kernels. Two hundred fifty cc of oat kernels per flask plus 125 ml of water were autoclaved at 121° C for 90 min on each of two consecutive days. The flasks were incubated at 25° C until the fungus colonized the oat kernels (about 3 wk). The oats were shaken once during the incubation period. After colonization, the oat kernels were removed from the flasks, dried and stored. The inoculum was pulverized using a Waring blender and sieved to obtain particles 0.25-0.5 mm in size. *G. g. tritici* inoculum was added to either steamed Ritzville silt loam or raw Puget silt loam (Pierson and Weller, *Phytopathology* 84:940-947 (1994)).

b. Preparation of bacterial treated seeds. Wheat seeds were surface-sterilized by immersion in a 2.6% solution of sodium hypochlorite for 2 min followed by a 10 min rinse under a stream of water and a final rinse of sterile water. Seeds were dried in a stream of sterile air. Wild-type strains (Q2-87, Q65c-80, and Q69c-80), strains transformed with Phl biosynthetic genes carried on the plasmid pVSP41 [(Q2-87(pPHL5122), Qc65(pPHL5122), and Q69c-80(pPHL5122)], strains bearing only the plasmid vector [Q65c-80(pVSP41) and Q69c-80(pVSP41)] and strain Q69c-80 transformed by Phl biosynthetic genes carried on a mini-transposon (Q69c-80::mTn5PHL12, Q69c-80::mTn5PHL15 and Q69c-80::mTn5PHL20) were cultured individually into 250-ml flasks containing 25 ml of nutrient broth yeast extract broth (Bacto-nutrient broth, 8 g; Bacto-yeast extract, 2 g; K₂HPO₄, 2 g KH₂PO₄, 0.5 g; MgSO₄ 7H₂O, 0.25 g; glucose, 5 g; H₂O, 1000 ml) Vidaver, (*Appl. Microbiol.* 15:1523-1524 (1967)) for 72 hr, centrifuged at 5,000 rpm for 10 min at 4°C, and suspended in 3 ml of 0.5% (w/w) of methylcellulose. Six grams of the surface-sterilized seeds were poured into the bacteria-methylcellulose mixture, shaken and then dried under a stream of sterile air. Seeds became coated with populations ranging from 4.0-9.0 log cfu/seed depending on the strain. A wide variety of microbiological media (both solid and liquid) can be substituted to culture the bacteria and a wide variety of

compounds can be substituted to formulate the bacteria and to apply them to the seeds (PCT Application WO 94/01561).

c. Growth chamber test using bacteria-treated seeds. Tests of bacterial strains and controls (nontreated seed or seed treated with methylcellulose) were conducted similar to tests reported by Ownley et al. (*Phytopathology* 82:178-184 (1992)). The test used plastic tubes (2.5-cm diameter x 16.5-cm long) held upright in plastic racks. The bottom of each tube was plugged with cotton and filled with a 6.5-cm-deep layer of sterile vermiculite overlaid with 10 g of soil amended with *G. g. tritici* oat kernel inoculum. Two wheat seeds treated with a bacterial strain or control seeds were sown per tube and covered with vermiculite. Each tube received 10 ml of water. The cones were incubated at 15-18° C for 3-4 weeks with a dark/light cycle of 12 hr and watered twice per week with 5 ml of dilute (1:3, v/v) Hoagland's solution (macroelements only) (Hoagland and Arnon, *J. Cal. Agr. Exp. Stat. Circ.* 347 (1950)). Each treatment was replicated five times, and each replicate consisted of a row of 10 separate tubes (20 individual plants). Treatments were arranged in racks in a randomized complete block design. After incubation, seedlings were removed from the soil, washed with water and evaluated on the basis of overall take-all severity and the number of root lesions using the following criteria. Overall take-all severity was measured on a scale of 0 (no disease) to 8 (plant nearly dead) as described by Ownley et al., (*Phytopathology* 82:178-184 (1992)). The severity of root disease (root disease index) was measured on the sections of the roots 3 cm or greater from the seed using a scale of 0-4, where 0 = no take-all lesions, 1 = up to 25% of the roots infected, 2 = 26%-50% of the roots infected, 3 = 51%-75% of the roots infected and 4 = over 75% of roots infected. Disease rating was compared statistically among treatments by an analysis of variance (ANOVA). Treatment means were compared by a least significant differences test (LSD) when significant differences among treatments was indicated by the ANOVA (Fisher's protected LSD). Differences among treatments were determine based on a probability level of $P=0.05$. Table 5 shows the enhanced biocontrol of take-all achieved by insertion of the DNA sequences that code for Phl production. For example, strain Q2-87(pPHL5122) had a significantly lower root disease index score than Q2-87.

Strain Q65c-80(pPHL5122) had a significantly lower overall disease severity rating than Q65c-80 or Q65c-80(pVSP41). Strain Q69c-80(pPHL5122) had a significantly lower overall disease severity rating than the wild-type strain Q69c-80 or Q69c-80(pVSP41). There was no soil x treatment interaction, therefore data from the raw Puget silt loam and pasteurized Ritzville silt loam were pooled.

Table 5

Seed treatment	Phl produced	Log cfu /seed	Overall disease severity	Root disease index
Non treated			3.56 a ^z	2.01 a ^z
Methylcellulose			3.53 a	1.94 a
Q2-87	+	7.9	3.09 b	1.22 b
Q2-87(pPHL5122)	++	5.6	3.03 b	0.89 c
Q65c-80	+	8.4	3.03 b	0.63 d
Q65c-80(pPHL5122)	++	8.0	2.75 cd	1.22 b
Q65c-88(pVSP41) (vector)	+	7.8	2.97 b	1.30 b
Q69c-80	-	8.5	2.95 bc	1.39 b
Q69c-80(pPHL5122)	++	8.8	2.61 d	1.19 b
Q69c-80(pVSP41) (vector)	-	9.1	2.93 bc	1.35 b

^z Means in the same column followed by the same letter are not significantly different at $P = 0.05$ according to Fishers' protected least significant difference test (LSD).

Table 6 shows that DNA sequences that code for Phl production, when stably inserted into the genome of the recipient strain, conferred on the recipient strain the ability to suppress take-all at a substantially lower bacterial dose on the seed as compared to the wild-type strain. For example, strains Q69c-80::mTn5PHL112, Q69c-80::mTn5PHL115 and Q69c-80::mTn5PHL20 applied at doses that were approximately 10,000-fold, 10,000-fold and 100-fold less, respectively than the dose of the wild-type strain Q69c-80 provided the same level of take-all suppression as Q69c-80. This

is important because each 10-fold reduction in the dose of the bacterial treatment results in a substantial reduction in the cost of the biocontrol treatment to the farmer. There was no soil x treatment interaction, therefore data for the raw Puget silt loam and pasteurized Ritzville silt loam were pooled.

Table 6

Seed treatment	Phl produced	Log cfu /seed	Overall disease severity
Methylcellulose			3.89 a ²
Q69c-80	-	8.6	3.03 b
Q69c-80::mTn5Phl12	++	4.1	3.16 b
Q69c-80::mTn5Phl15	++	4.4	3.19 b
Qc69c-80::mTn5Phl20	++	6.1	2.95 b

² Means in the same column followed by the same letter are not significantly different at $P = 0.05$ according to Fishers' protected least significant difference test (LSD).

Example 9. Field testing of strains transformed with DNA sequences that confer of enhance Phl production.

a. Preparation of bacterial treated seeds. Wild-type strains Q2-87, Q65c-80, and Q69c-80), strains transformed with Phl biosynthetic genes carried on the plasmid pVSP41 [(Q2-87(pPHL5122), Qc65(pPHL5122), and Q69c-80(pPHL5122)], strains bearing only the plasmid vector [Q65c-80(pVSP41) and Q69c-80(pVSP41)] and strain Q69c-80 transformed by Phl biosynthetic genes carried on a mini-transposon Q69c-80::mTn5PHL15 and Q69c-80::mTn5PHL20) are propagated for 48-72 hr individually on agar plates of King's Medium B (Proteose peptone, 20 g; K_2HPO_4 , 1.5 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; glycerol, 10 g; agar, 17 g; H_2O , 1000 ml) (King et al., *J. Lab. Clin. Med.* 44:301-307 (1954)). The bacteria are scraped from the plates and suspended in 1.0% methylcellulose.

Bacteria are mixed with seed at a rate of approximately one petri plate of bacteria and 4.0 ml of methylcellulose per 20 g of seed. Coated seeds are air dried and contain approximately 4.0-8.5 log cfu/seed depending on the strain. A wide variety of microbiological media (both solid and liquid) can be substituted to culture the bacteria and a wide variety of compounds can be substitute to formulate the bacteria and apply them to the seeds (see PCT Application WO 94/01561).

Strains are tested in fields in which *G. g. tritici* is introduced as oat kernel inoculum. Treatments are arranged in a highly modified randomized complete block design as described by Pierson and Weller (supra). Seed treatments and control treatments each are sown in three 3.1-m rows. *G. g. tritici*, as whole oat kernel inoculum is introduced into the furrow immediately before the seed is sown by hand. Controls include methylcellulose-treated seed with *G. g. tritici* inoculum in the furrow, nontreated seed with *G. g. tritici* in the furrow, and nontreated seed without *G.g. tritici*. Strains also are tested in fields that are naturally infested with *G. g. tritici*. In such tests, controls consist only of nontreated seed and methylcellulose treated seed. The severity of take-all is assessed on the basis of plant height, the number of heads, the amount of root disease and the grain yield.

Example 10. Use of strains transformed with DNA sequences that confer or enhance Phl production to suppress take-all in commercial wheat fields.

Bacterial strains transformed with Phl-biosynthetic sequences are cultured as described in Examples 8 and 9 or by any commercial method of bacterial mass rearing and are applied to wheat seed directly or introduced into the seed furrow, for example as a spray at log 6-9 cfu/ml, at the time of planting in fields infested with *G. g. var tritici* in order to suppress take-all. Field preparation and seeding follows standard commercial practices and uses standard commercial equipment. The effectiveness of the bacterial treatment is determined as described in Example 9.

Example 11. Use of strains transformed with DNA sequences that

confer or enhance Phl production to suppress pathogens of wheat and barley in fields.

Bacterial strains transformed with Phl-biosynthetic sequences, cultured as described in Examples 8 and 9, are applied to wheat or barley seed directly or introduced into the seed furrow at the time of planting in field infested with any wheat pathogen, for example, those belonging to the genera *Gaeumannomyces*, *Pythium*, *Fusarium*, *Rhizoctonia*, *Tilletia*, *Urocystis*, *Cephalosporium*, *Microdochium*, *Typhula*.

Example 12. Use of strains transformed with DNA sequences that confer or enhance Phl production to suppress pathogens that cause seed rot, damping-off, blights, root rots, wilts and crown rots of crops grown from seeds, cuttings or seedpieces.

Bacterial strains transformed with Phl-biosynthetic sequences are cultured as described in Examples 8 and 9 and applied to seeds, cuttings, and seedpieces of crops infected with any pathogens, for example belonging to the genera *Fusarium*, *Rhizoctonia*, *Pythium*, *Phytophthora*, *Verticillium*, *Septoria*, that cause seed rot, damping-off, blights, root rots, wilts and crown rots.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) INVENTORS: Thomashow, Linda S
Bangera, Mahalaxmi
Weller, David M
Cook, R. James
- (ii) TITLE OF INVENTION: Sequences for Production of
2,4-Diacetylphloroglucinol and Methods
- (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Stephan A. Pendorf, DOMINIK & STEIN
 - (B) STREET: 600 N. West Shore Boulevard, #1000
 - (C) CITY: Tampa
 - (D) STATE: FL
 - (E) COUNTRY: USA
 - (F) ZIP: 33609
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Pendorf, Stephan A.
 - (B) REGISTRATION NUMBER: 32665
 - (C) REFERENCE/DOCKET NUMBER: A700.320
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (813) 289-2966
 - (B) TELEFAX: (813) 289-2967

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3680 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

- (vi) ORIGINAL SOURCE:
 (A) ORGANISM: *Pseudomonas fluorescens*
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 (D) OTHER INFORMATION: /note= "phlR, transcribed from left to right"
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5076 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

(A) NAME/KEY: misc feature
 (B) LOCATION: complement (4285..5076)
 (D) OTHER INFORMATION: /note= "phlA, transcribed from
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 (D) OTHER INFORMATION: /note= "phlD, transcribed from
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GTTGTCAGCC ACATCGGCGG CGCAAGAAAA GCTGGCATCG AAATGCGCGA TCACATTTTC 4860
AGTACCCAAC AACAGTGCGG CGGCTCCCGC TCCGGCGTAG GACTCGGTCA AGTCGCCGGG 4920
GGCGGTGTTG CGGTTGATCG TATCGGCGCC TATTGCCAGT GCATTGCCGG CCATGCCCGA 4980
GGCTACCAGG GCATAGGCGA TCTGCAGGGC GCTGGTGCCT GATTGCCGG CAAACTGTAC 5040
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6387 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: complement (4285..5076)
- (D) OTHER INFORMATION: /note= "phlA, transcribed from right to left"

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: complement (2606..3313)
- (D) OTHER INFORMATION: /note= "phlB, transcribed from right to left"

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: complement (308..4251)
- (D) OTHER INFORMATION: /note= "phlC, transcribed from right to left"

- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: complement (1398..2444)
 (D) OTHER INFORMATION: /note= "phlD, transcribed from right to left"
- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 3514..4767
 (D) OTHER INFORMATION: /note= "phlR, transcribed from left to right"
- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: complement (2..1270)
 (D) OTHER INFORMATION: /note= "phlE, transcribed from right to left"
- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 5781..6386
 (D) OTHER INFORMATION: /note= "phlF, transcribed from left to right"
- (ix) FEATURE:
 (A) NAME/KEY: misc_feature
 (B) LOCATION: 1..6387
 (D) OTHER INFORMATION: /note= "SEQ ID NO:3 contains genes involved in synthesis and modulation of synthesis of Phl."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CAGAGCTGGC TTCCTTGAGC CGCAGGGACA GAAACCCGCC CATCAAAC TA CCAGCCAGTG	120
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CAACGCCACC GCCCAGGATT TCTCCGCAAC CCACCACCAG GCCCGTAGCG GTGGCCAGTA	240
AACTGGGTGG CACTGATTCTG CTGGTCAGTG GGCCGACGGT GATGCAGATC AGGCTGAAAT	300
TGATGAAAGA TAAAAAGAAC AGCTGGAGGA ACAGTAGCCA CGGTAACGGC GGGGAAATGA	360
TGAGCAAGCC GACCAGTAGT GTGCTGATCA GGAAGCAGAT GGAAACGACA GGCTTGCGGC	420
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CGGAGATGAT CATGGCCATG GAGAAATTGC TCAGGTGCAA GACGTCTGTC AGGTAGCTGG	540
GGAGCAGGGC GCACAGGACG AATTGGCAGC TCAGTATGCA TAGCATCAAG GCAATGTTGA	600
GGCGCACGTT GCCGCTGGAC AGGGCTGTTC GCCATTGGCT GCCGGAGGGT TCTACGAGCG	660
GCCTTGATG GGGCGCCTGG CTCGGTTGGT AGGTTGATA CAGATACCAG GCCACCAGCA	720
GGCCCGGCAA CGAGATGATG GCGAACACGG CGCGCCACGA TCCGAACATT TCAAACAATA	780
CGCCCGCCAG CAGCGGCCCC AGGCACAGGC CGATGATGGG AAACAGTGCC TGCTGGATGC	840

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CAAATGCCAG GATCGCCGAT AACAGGCCCA GGTCTTGATA GTCCAGGGCC AGGTCATGCA 1140
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GGCAAGACCA GATACCGTTC ATTGACTTGG GTGTTTTGAA TCATCCGTTT AGCCAATGCC 2340
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TTGTAATGCG GGAAAAGCAA GCTGGGTTTG CAAAGAGTAG ACATGACAAG TCCTCGGCTG 2460
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AATGCGAGCG	TCCTTGACTT	GGTTGTTGGA	CTCGCCGCGA	AGTTGTCGGA	ACAGCTCGGT	3180
AATGTGCAGG	ATGCCGTCGC	AACCAGAGGC	GTGGCCGCGG	CCAATATTGC	CGCCATCGGT	3240
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GACTTGGGCC	ACATCGATAT	CCTTGGCGGT	GATGCCGGCT	TTTTTGTAGG	CGATTTCCGGC	3420
GCAAGCAATG	GAGTTGGCGG	AAACCGCCAT	GCCGACGTCT	TTTGGCAGGC	CTGGATATTT	3480
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GCGCATTTGC	CGACCAAACG	TTGCCAGGAC	ATCCGCTTCG	CGTCCTGCAT	AGCCAAATTT	3780
TTCAAAATAT	TCGGCGGTTG	CGAGGGCAAA	GGCGTCGGTG	TGCGAAATGC	CCAGGAAATA	3840
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GCTGATGCCC	AGGGCATCGG	AAATAGTCGG	GCCCAGGCCG	CCGTATTCGG	AAATACCTTC	4080
ACCGTGATAT	CCATAAGCGA	CTGCCTGAAG	TTCACGGGGA	TGCATCTTGA	TGGCGTTGAG	4140
CGCCTGATAG	GCGGACTCGA	CGATCATCTC	CTTGAAGGTT	TGACGGACTC	TGGAGCTGCC	4200
GGGTTTGGA	GTATAGGCAG	CCGAAACGAT	AGCAACGCGT	CGTGCGCTCA	TTGGAAGTGC	4260
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TTTGAACTCG	TACTTGATCG	ACGTCCCGTA	ATCCACGTAA	TACTTGCTTT	CCAGCAGCGT	4380
GCGCAGCGCA	ACGTTGGTCT	TTTGGTAGGC	TTGATGGCA	TCGGTCACTG	TCAACGCAAT	4440
CGCATCGCTG	CCCGCACCAA	ACCCGTACGA	CACCA ² AGG	ATTTTTTCAC	CCGGACGCGC	4500
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GAAGGGGGTC GAAACCAGGT TTTGCTGGAA TACGACATAG TCGAAATCGC TGGCCTGTAC	4680
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GTTGTCAGCC ACATCGGCGG CGCAAGAAAA GCTGGCATCG AAATGCGCGA TCACATTTTC	4860
AGTACCCAAC AACAGTGCGG CGGCTCCCGC TCCGGCGTAG GACTCGGTCA AGTCGCCGGG	4920
GGCGGTGTTG CGGTTGATCG TATCGGCGCC TATTGCCAGT GCATTGCCGG CCATGCCCGA	4980
GGCTACCAGG GCATAGCGA TCTGCAGGGC GCTGGTGCCT GATTTGCCGG CAAACTGTAC	5040
GTCCGCGCAG AAGGCGTCAT AACCGCAGCC GAGCATTTCC AGAATGACCG CGGCCGAGGC	5100
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GGCTTTGTCC AGCGCACGTT GAGCGGCCAG GACACTCAAG GTAATGACGT CCTCATCGGG	5220
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TTTCCAGACG TGGATCACGT CTTCCACTTT AAGGCGGCAC ACCGGGATGC CCGCGCCATA	5340
GCTCACAATT CCTACTTTAT TCACGTGTAC TTCCTCCAGA TTCCTTTCTT CACCTGCCAG	5400
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GAGGGAGAAC GGGTTTATTA TCCTTCTATT ATTATGTATG ATACGAAACG TGCCGTATCG	5580
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GGAGCCCACA TACGCACAAA GCGATCATCA TCTCCGCTAT AGAAACACTC AAGGAGTGCG	5880
GTTATTCAGG GTTGAGTATC GAGGCTGTGG CTCGCCGTGC CGGCGCGAGC AAGCCGACCA	5940
TCTATCGATG GTGGGGTAAC AAGGCGGCTT TGATCGCCGA AGTCTACGAG AGCGAAAGCG	6000
AGCAGATTCG CAAGGAGCCT GATAAAGGAT CCTTCAAGGA GAACCTCAAT TTCCTGCTGC	6060
TCAATCTGTG GAAGGTCTGG AGAGAAACGA TTTGCGGGGA GCGGTTTCGG TGTGTCATCG	6120
CTGAAGCCCA GCTCGACCCC AGTACGCTGC CCAAGCTGAA GGATGAATTC ATGGAGCGTC	6180
GTCGGGAATT GCCGCGAAAG CTGGTGGAAA ACGCCATCCA GCAAGGTGAG TTGCCCAGG	6240
ACACGTCCCG TGAGTTGTTG TTGGACATGA TCTTCGGATT TTGCTGGTAC AGGCTGTTGA	6300
CTGAGCAACT GGAAGTGGAG GGTGACATCA ATGAATTCAC GACGCTTCTG TTGAACGGCG	6360
TGTTGCGTAC GACTTCGGCG GCGGAGT	6387

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6170 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Pseudomonas fluorescens
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: complement (4285..5076)
 - (D) OTHER INFORMATION: /note= "phlA, transcribed from right to left"
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: complement (2606..3313)
 - (D) OTHER INFORMATION: /note= "phlB, transcribed from right to left"
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: complement (3085..4251)
 - (D) OTHER INFORMATION: /note= "phlC, transcribed from right to left"
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: complement (1398..2444)
 - (D) OTHER INFORMATION: /note= "phlD, transcribed from right to left"
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 3514..4767
 - (D) OTHER INFORMATION: /note= "phlR, transcribed from left to right"
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: complement (2..1270)
 - (D) OTHER INFORMATION: /note= "phlE, transcribed from right to left"
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 5781..6170
 - (D) OTHER INFORMATION: /note= "phlF, truncated, transcribed from left to right"
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 1..6170

(D) OTHER INFORMATION: /note= "SEQ ID NO:4 contains genes involved in synthesis, and modulation of synthesis of Phl."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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CAACGCCACC GCCCAGGATT TCTCCGCAAC CCACCACCAG GCCCGTAGCG GTGGCCAGTA	240
AACTGGGTGG CACTGATTCG CTGGTCAGTG GGCCGACGGT GATGCAGATC AGGCTGAAAT	300
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GGCGCACGTT GCCGCTGGAC AGGGCTGTTC GCCATTGGCT GCCGGAGGGT TCTACGAGCG	660
GCCTTGATG GGGCGCCTGG CTCGGTTGGT AGGTTGATA CAGATACCAG GCCACCAGCA	720
GGCCCCGCAA CGAGATGATG GCGAACACGG CGCGCCACGA TCCGAACATT TCAAACAATA	780
CGCCCCCAG CAGCGGCCCC AGGCACAGGC CGATGATGGG AAACAGTGCC TGCTGGATGC	840
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CAAATGCCAG GATCGCCGAT AACAGGCCCA GGTCTGATA GTCCAGGGCC AGGTCATGCA	1140
TGATCACCGG GAACAACGGC ATGATAATGA ATCGATCAAG TCCTACCAGC CCGAAGCTCA	1200
GCGACAAAAG AACGACCATG CGTCTTTCGT AGCCACCCCA AGGTCGAGTG GCAAGATACG	1260
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TGAGCGATCG	GCAATTGTAC	GGTCGACGTT	CGCAGTCCCA	AGTCATTGAT	CAGGTGGGCT	2100
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TCCTTGCTGG	ATGGTTGGGA	ATCAGAGGTA	GGCTGTCAGG	GCGTAGTCAG	GCCGCAAGTA	4320
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CGCATCGCTG	CCCGCACCAA	ACCCGTACGA	CACCAAGAGG	ATTTTTTTCAC	CCGGACGCGC	4500
TCGGTCCAGT	ACGCTCACCA	AGCCCAGCAA	CGGACTCGCG	GGCCCCGCAT	CACCGACACT	4560
CTGGGCATAA	ATGCCAGGTT	CGATCTGCGC	TTTGGTGAAG	CCCAGGCCTT	TGCCAAGAGA	4620
GAAGGGGGTC	GAAACCAGGT	TTTGCTGGAA	TACGACATAG	TCGAAATCGC	TGGCCTGTAC	4680
ATTCATCTTG	GCCATCAATC	CCGACGCAGC	ACGATGGGTC	TGGTCTTCAA	GGCCAATGCT	4740
GTTCTTGTCG	GAGCCCAGCC	CCATTCCCTGA	GCGAATGTAG	CGGTCTCCCT	GGGGGCGGAT	4800
GTTGTCAGCC	ACATCGGCGG	CGCAAGAAAA	GCTGGCATCG	AAATGCGCGA	TCACATTTTC	4860
AGTACCCAAC	AACAGTGCGG	CGGCTCCCGC	TCCGGCGTAG	GACTCGGTCA	AGTCGCCGGG	4920
GGCGGTGTTG	CGGTTGATCG	TATCGGCGCC	TATTGCCAGT	GCATTGCCGG	CCATGCCCGA	4980
GGCTACCAGG	GCATAGGCGA	TCTGCAGGGC	GCTGGTGCCT	GATTTGCCGG	CAAACGTGAC	5040
GTCCGCGCAG	AAGGCGTCAT	AACCGCAGCC	GAGCATTTCC	AGAATGACCG	CGGCCGAGGC	5100
GCGGGAGTCA	TATGGGTTGG	TGCACGTACC	CAGGTACAGC	GCTTCCAGGT	CGCAAGAAGG	5160
GGCTTTGTCC	AGCGCACGTT	GAGCGGCCAG	GACACTCAAG	GTAATGACGT	CCTCATCGGG	5220
TTGGAGTACA	GCCCTTTCAA	CGACGCCCAG	TTGGTTGGTG	ACCAGACTCA	AGTCTGTGTT	5280
TTTCCAGACG	TGGATCACGT	CTTCCACTTT	AAGGCGGCAC	ACCGGGATGC	CCGCGCCATA	5340
GCTCACAATT	CCTACTTTAT	TCACGTGTAC	TTCCTCCAGA	TTCCTTTCTT	CACCTGCCAG	5400


```

CGGATAGCCG TGACCGATGC ATGAAATATT TAGAAACTAT CTAACGGTGC CCGCAAAGTG 5460
TCGTTGGCAG TCCTATGCCC GGAAATCGGG CTCCTCAAGG GGGAAACTA CAGTTCCTTT 5520
GAGGGAGAAC GGGTTTATTA TCCTTCTATT ATTATGTATG ATACGAAACG TGCCGTATCG 5580
TTAAGGTCTT GTTAAAAATT GATGACTATT TATCGGGTTT CTCCTATCT AGTGGCAAGT 5640
TCCGCTATTG AGGTGTGCAG TTAAGCAGAA ACTTAGATCA TAAAAACATA CCAAACGAA 5700
ACGATCCGTT TCATTGCTTT TCGAGAGAAT CCTATACCTT GCGTCTCTTT TGTCAAGCGC 5760
CATATTGGAG ATTTTGAATT ATGGCCCGTA AACCGTCTCG GAGCTCCATT GGCTCATTGA 5820
GGAGCCCACA TACGCACAAA GCGATCATCA TCTCCGCTAT AGAAACACTC AAGGAGTGCG 5880
GTTATTCAGG GTTGAGTATC GAGGCTGTGG CTCGCCGTGC CGGCGCGAGC AAGCCGACCA 5940
TCTATCGATG GTGGGGTAAC AAGGCGGCTT TGATCGCCGA AGTCTACGAG AGCGAAAGCG 6000
AGCAGATTCG CAAGGAGCCT GATAAAGGAT CCTTCAAGGA GAACCTCAAT TTCCTGCTGC 6060
TCAATCTGTG GAAGGTCTGG AGAGAAACGA TTTGCGGGGA GGC GTTTCGG TGTGTCATCG 6120
CTGAAGCCCA GCTCGACCCC AGTACGCTGC CCAAGCTGAA GGATGAATTC 6170

```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 792 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: complement (1..792)
- (D) OTHER INFORMATION: /note= "phlA DNA sequence. SEQ ID NO:6 is translation (protein) of SEQ ID NO:5."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

GAGGTAGGCT GTCAGGGCGT AGTCAGGCCG CAAGTATTTG AACTCGTACT TGATCGACGT 60
CCCGTAATCC ACGTAATACT TGTCTTCCAG CAGCGTGCGC AGCGCAACGT TGGTCTTTTG 120
GTAGGCTTCG ATGGCATCGG TCACTGTCAA CGCAATCGCA TCGCTGCCCC CACCAAACCC 180
GTACGACACC AAGAGGATTT TTTCACCCGG ACGCGCTCGG TCCAGTACGC TCACCAAGCC 240
CAGCAACGGA CTCGCGGGCC CCGCATCACC GACACTCTGG GCATAAATGC CAGGTTCGAT 300

```

```

CTGCGCTTTG GTGAAGCCCA GGCCTTTGCC AAGAGAGAAG GGGGTCGAAA CCAGGTTTTG      360
CTGGAATACG ACATAGTCGA AATCGCTGGC CTGTACATTC ATCTTGGCCA TCAATCCCGA      420
CGCAGCACGA TGGGTCTGGT CTTCAAGGCC AATGCTGTTC TTGTCGGAGC CCAGCCCCAT      480
TCCTGAGCGA ATGTAGCGGT CTCCCTGGGG GCGGATGTTG TCAGCCACAT CGGCGGCGCA      540
AGAAAAGCTG GCATCGAAAT GCGCGATCAC ATTTTCAGTA CCCAACAACA GTGCGGCGGC      600
TCCCCTCCG GCGTAGGACT CCGTCAAGTC GCCGGGGGCG GTGTTGCGGT TGATCGTATC      660
GGCGCCTATT GCCAGTGCAT TGCCGGCCAT GCCCAGGCT ACCAGGGCAT AGGCGATCTG      720
CAGGGCGCTG GTGCCTGATT TGCCGGCAAA CTGTACGTCC GCGCAGAAGG CGTCATAACC      780
GCAGCCGAGC AT                                                                792

```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 264 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Leu Gly Cys Gly Tyr Asp Ala Phe Cys Ala Asp Val Gln Phe Ala
 1           5           10           15
Gly Lys Ser Gly Thr Ser Ala Leu Gln Ile Ala Tyr Ala Leu Val Ala
          20           25           30
Ser Gly Met Ala Gly Asn Ala Leu Ala Ile Gly Ala Asp Thr Ile Asn
          35           40           45
Arg Asn Thr Ala Pro Gly Asp Leu Thr Glu Ser Tyr Ala Gly Ala Gly
          50           55           60
Ala Ala Ala Leu Leu Leu Gly Thr Glu Asn Val Ile Ala His Phe Asp
          65           70           75           80
Ala Ser Phe Ser Cys Ala Ala Asp Val Ala Asp Asn Ile Arg Pro Gln
          85           90           95
Gly Asp Arg Tyr Ile Arg Ser Gly Met Gly Leu Gly Ser Asp Lys Asn
          100          105          110
Ser Ile Gly Leu Glu Asp Gln Thr His Arg Ala Ala Ser Gly Leu Met
          115          120          125
Ala Lys Met Asn Val Gln Ala Ser Asp Phe Asp Tyr Val Val Phe Gln
          130          135          140
Gln Asn Leu Val Ser Thr Pro Phe Ser Leu Gly Lys Gly Leu Gly Phe
          145          150          155          160
Thr Lys Ala Gln Ile Glu Pro Gly Ile Tyr Ala Gln Ser Val Gly Asp
          165          170          175

```

Ala Gly Pro Ala Ser Pro Leu Leu Gly Leu Val Ser Val Leu Asp Arg
 180 185 190

Ala Arg Pro Gly Glu Lys Ile Leu Leu Val Ser Tyr Gly Phe Gly Ala
 195 200 205

Gly Ser Asp Ala Ile Ala Leu Thr Val Thr Asp Ala Ile Glu Ala Tyr
 210 215 220

Gln Lys Thr Asn Val Ala Leu Arg Thr Leu Leu Glu Asp Lys Tyr Tyr
 225 230 235 240

Val Asp Tyr Gly Thr Ser Ile Lys Tyr Glu Phe Lys Tyr Leu Arg Pro
 245 250 255

Asp Tyr Ala Leu Thr Ala Tyr Leu
 260

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 708 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: complement (1..708)
- (D) OTHER INFORMATION: /note= "phlB DNA sequence. SEQ ID NO:8 is translation (protein) of SEQ ID NO:7."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

TTTCGCCAAT ACGAATTTAT AAGCGTATTG CCACGCCAGG TTGCTTTCCC GAACGTGCTT      60
GCGAATAACC ATTGCGACTG GTGCTCCAGT CACGACTTGC CGGGGATCGA CGACATCGAC      120
GATTTCCGAG GCGATCACCA AGCCATCGTC CAGGCGCACC ATTGCCATGA AGCGCGGGAC      180
GGTTTCGCCA TATCCCATGG CCGCGAGAAT GGGGTTTTTCA GCATGGGCGC TGACCTGGAT      240
CGTGCCGGTG CGTGCGCAGC GATACGGTTC CACGTTCAAT GAGTTGCATG CGCCGCAGAC      300
GGTGCGCCGT GGGAAGAAGA TTTCTTCGCA ATCCTGGCAG CGGCTGCCTT CGAGACGATA      360
TTTTCCGCCA TGTTGCGGCC ATTCGCGCAA CATGCTGGCG GTGGTCATGC GGTGTATTTG      420
TTCTGGGTAA AGGGACATGG TCGGCTCCTT AATCGTTGGA AAGCACAATG ACGCTGTTAT      480
GCGCGGCGTA ACCGCCAAG TTCCTGCGAG ACGCCAATGC GAGCGTCCTT GACTTGGTG      540

```

TTGGA CTGCG CGCGAAGTTG TCGGAACAGC TCGGTAATGT GCAGGATGCC GTCGCAACCA 600
 GAGGCGTGGC CGCGGCCAAT ATTGCCGCCA TCGGTGTTTA ATGGCAGTTG CCCGTCGAGG 660
 GCTATGCCGC CTTCCAATAC AAAGTCGCCT GCCTGGCCTG GACCACAT 708

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 236 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Trp Ser Arg Pro Gly Arg Arg Leu Cys Ile Gly Arg Arg His Ser
 1 5 10 15
 Pro Arg Arg Ala Thr Ala Ile Lys His Arg Trp Arg Gln Tyr Trp Pro
 20 25 30
 Arg Pro Arg Leu Trp Leu Arg Arg His Pro Ala His Tyr Arg Ala Val
 35 40 45
 Pro Thr Thr Ser Arg Arg Val Gln Gln Pro Ser Gln Gly Arg Ser His
 50 55 60
 Trp Arg Leu Ala Gly Thr Trp Ala Val Thr Pro Arg Ile Thr Ala Ser
 65 70 75 80
 Leu Cys Phe Pro Thr Ile Lys Glu Pro Thr Met Ser Leu Tyr Pro Glu
 85 90 95
 Gln Ile His Arg Met Thr Thr Ala Ser Met Leu Arg Glu Trp Arg Glu
 100 105 110
 His Gly Gly Lys Tyr Arg Leu Glu Gly Ser Arg Cys Gln Asp Cys Glu
 115 120 125
 Glu Ile Phe Phe Pro Arg Arg Thr Val Cys Gly Ala Cys Asn Ser Leu
 130 135 140
 Asn Val Glu Pro Tyr Arg Cys Ala Arg Thr Gly Thr Ile Gln Val Ser
 145 150 155 160
 Ala His Ala Glu Asn Pro Ile Leu Ala Ala Met Gly Tyr Gly Glu Thr
 165 170 175
 Val Pro Arg Phe Met Ala Met Val Arg Leu Asp Asp Gly Leu Val Ile
 180 185 190
 Ala Ser Glu Ile Val Asp Val Val Asp Pro Arg Gln Val Val Thr Gly
 195 200 205
 Ala Pro Val Arg Met Val Ile Arg Lys His Val Arg Glu Ser Asn Leu
 210 215 220
 Ala Trp Gln Tyr Ala Tyr Lys Phe Val Leu Ala Lys
 225 230 235

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1167 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: complement (1..1167)
- (D) OTHER INFORMATION: /note= "phlC DNA sequence. SEQ ID NO:10 is translation (protein) of SEQ ID NO:9."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

TGCGCGGCGT AACCGCCCAA GTTCCTGCGA GACGCCAATG CGAGCGTCCT TGA CTTGGTT      60
GTTGGACTCG CCGCGAAGTT GTCGGAACAG CTCGGTAATG TGCAGGATGC CGTCGCAACC      120
AGAGGCGTGG CCGCGGCCAA TATTGCCGCC ATCGGTGTTT AATGGCAGTT GCCCGTCGAG      180
GGCTATGCCG CCTTCCAATA CAAAGTCGCC TGCCTGGCCT GGACCACATA CGCCCATGGA      240
TTCCATCTGA ATCAATCCGG CACCCAGCAA GTCGTAGACT TGGGCCACAT CGATATCCTT      300
GGCGGTGATG CCGGCTTTTT TGTAGGCGAT TTCGGCGCAA GCAATGGAGT TGGCGGAAAC      360
CGCCATGCCG ACGTCTTTTG GCAGGCCTGG ATATTT CAGG GTCGGGTTGT GATAGCGCGT      420
CCCGAAATAA TGGGATACGC CGGTATAGGC ACAACCACGG ACGAATACCG GTTGGGTCGT      480
GTAGCGGTGC GCCAGGTGTT CGGCGACCAG GATGGCGCAA CCGCTGGCTT CACCCCAGGC      540
CAGCATCGAG CCACATGCTT CGCTGTTCTT GAGGGTTTCA AGGGATGGCA CCGGCACGCC      600
ATAGCGGGTT GCCGTGGGCG TGTGTGCGC ATAGATGCGC ATTTGCCGAC CAAACGTTGC      660
CAGGACATCC GCTTCGCGTC CTGCATAGCC AAATTTTCA AAATATTCGG CGGTGCGAG      720
GGCAAAGGCG TCGGTGTGCG AAATGCCAG GAAATAATCG TACTCACATT CGGTACTGGA      780
GCCGATGTAT TCGGCATAGT TGAAGTGGTC GGTCATTTTT TCAAAGCCAC CACACAGGAC      840
GATGTCGTAC TCACCCGAGG CGACCATCTG ATGGGCCATC TGAAAGGAAA CCGAGCTGCT      900
GGTGCAGTTG GCAGTGCTCA TGAACGTCGG GGCAGGGCTG ATGCCAGGG CATCGGAAAT      960
AGTCGGGCCC AGGCCGCCGT ATTCGGAAAT ACCTTCACCG TGATATCCAT AAGCGACTGC      1020
CTGAAGTTCA CGGGGATGCA TCTTGATGGC GTTGAGCGCC TGATAGGCGG ACTCGACGAT      1080

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CATCTCCTTG AAGGTTTGAC GGACTCTGGA GCTGCCGGGT TTGGAAGTAT AGGCAGCCGA 1140
AACGATAGCA ACGCGTCGTG CGCTCAT 1167

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 389 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Ser Ala Arg Arg Val Ala Ile Val Ser Ala Ala Tyr Thr Ser Lys
1 5 10 15
Pro Gly Ser Ser Arg Val Arg Gln Thr Phe Lys Glu Met Ile Val Glu
20 25 30
Ser Ala Tyr Gln Ala Leu Asn Ala Ile Lys Met His Pro Arg Glu Leu
35 40 45
Gln Ala Val Ala Tyr Gly Tyr His Gly Glu Gly Ile Ser Glu Tyr Gly
50 55 60
Gly Leu Gly Pro Thr Ile Ser Asp Ala Leu Gly Ile Ser Pro Ala Pro
65 70 75 80
Thr Phe Met Ser Thr Ala Asn Cys Thr Ser Ser Ser Val Ser Phe Gln
85 90 95
Met Ala His Gln Met Val Ala Ser Gly Glu Tyr Asp Ile Val Leu Cys
100 105 110
Gly Gly Phe Glu Lys Met Thr Asp His Phe Asn Tyr Ala Glu Tyr Ile
115 120 125
Gly Ser Ser Thr Glu Cys Glu Tyr Asp Tyr Phe Leu Gly Ile Ser His
130 135 140
Thr Asp Ala Phe Ala Leu Ala Thr Ala Glu Tyr Phe Glu Lys Phe Gly
145 150 155 160
Tyr Ala Gly Arg Glu Ala Asp Val Leu Ala Thr Phe Gly Arg Gln Met
165 170 175
Arg Ile Tyr Ala His Asn Thr Pro Thr Ala Thr Arg Tyr Gly Val Pro
180 185 190
Val Pro Ser Leu Glu Thr Leu Lys Asn Ser Glu Ala Cys Gly Ser Met
195 200 205
Leu Ala Trp Gly Glu Ala Ser Gly Cys Ala Ile Leu Val Ala Glu His
210 215 220
Leu Ala His Arg Tyr Thr Thr Gln Pro Val Phe Val Arg Gly Cys Ala
225 230 235 240

(2) INFORMATION FOR SEQ ID NO:11:

(A) LENGTH: 1047 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: complement (1..1047)
(D) OTHER INFORMATION: /note= "phlD DNA sequence. SEQ ID NO:12 is translation (protein) of SEQ ID NO:11."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCCACCCAC TTGCCCACGG CCATTTCAGC TGTGAAGCCA GGGCCGAAGG CTGCCAGCAT 60
GCCGGTCGCT CCATTGGCCG GCCCGCTGTC GAACTGGCGC TTGAGGACGT CGAAGACCAC 120
CACGCTGGCA ATATTGCCGG CCTCGCTCAA GCTGTCGCGA GACTGCGCGA CCCTGCCAGG 180

```

TTCCAGATCG AGCTGCAGCA CCAGCTCATC AAGAATTTTT CGTCCACCGG TGTGGAAGAT      240
GAAAAAGTCA TTTTGAGCGC AATGTTGGTT GAAGGTCTCG AAGTTCAATT CCTCCATCAT      300
CGGGGCCACG TCTTTAATGG AGTTCATGAC GGCTTTGTCC AGGGTGAAGT GAAAGCCGCT      360
GTCCTTGACG TCATATTTAA TGTAGTGCTC GCTGTCAGGC AGGAAATAAG AGCCGGTTTT      420
GGCGATCTTG AATCCCGGCG CCTTATCGTC GGCGCGCATT ACGCAGGCCG AGACGGCATC      480
GCCGAACAGC GCTGCGGATA TGAACGCGTG CAACTTGGTG TCCTGTGGTT GATAGCAGAG      540
TGACGAGAAC TCCAGCGAGA CAATAAGGGC GTGGTTGTCT GGAGACAGGC TGCCAAAGTC      600
GTTGGCTCGA TTAATCGCCG CGGCGCCTGC CACGCATCCC AATTGAGCGA TCGGCAATTG      660
TACGGTCGAC GTTCGCAGTC CCAAGTCATT GATCAGGTGG GCTGTCAGCG ATGGCATCAT      720
GAACCCGGTG CAAGAGGTAA CGGCGACCAT CCGGATGTCG TCCGTGGTCA AGCCCGCGTT      780
TTCAATGGCC TGGCGCGCGG CGATTGAAGA CATGCGGCGA GCCTCTCGCT CATAACGAT      840
GCTGCGGTGG GTAAAGCCGG TATGCACCGC AAGTTCATCG ATGGGCAAGA CCAGATACCG      900
TTCATTGACT TGGGTGTTTT GAATCATCCG TTTAGCCAAT GCCATGCGCG GATGATCGTC      960
ATGCAACTGT TCCAAGTGAT CGATCATCTG TTGTTGGGTA ATTTTGTAAT GCGGGAAAAG     1020
CAAGCTGGGT TTGCAAAGAG TAGACAT                                           1047

```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 349 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Met Ser Thr Leu Cys Lys Pro Ser Leu Leu Phe Pro His Tyr Lys Ile
 1              5              10              15
Thr Gln Gln Gln Met Ile Asp His Leu Glu Gln Leu His Asp Asp His
 20              25              30
Pro Arg Met Ala Leu Ala Lys Arg Met Ile Gln Asn Thr Gln Val Asn
 35              40              45
Glu Arg Tyr Leu Val Leu Pro Ile Asp Glu Leu Ala Val His Thr Gly
 50              55              60
Phe Thr His Arg Ser Ile Val Tyr Glu Arg Glu Ala Arg Arg Met Ser
 65              70              75              80
Ser Ile Ala Ala Arg Gln Ala Ile Glu Asn Ala Gly Leu Thr Thr Asp
 85              90              95
Asp Ile Arg Met Val Ala Val Thr Ser Cys Thr Gly Phe Met Met Pro
100            105            110

```



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Ser Leu Thr Ala His Leu Ile Asn Asp Leu Gly Leu Arg Thr Ser Thr
   115                               120                               125

Val Gln Leu Pro Ile Ala Gln Leu Gly Cys Val Ala Gly Ala Ala Ala
   130                               135                               140

Ile Asn Arg Ala Asn Asp Phe Gly Ser Leu Ser Pro Asp Asn His Ala
  145                               150                               155                               160

Leu Ile Val Ser Leu Glu Phe Ser Ser Leu Cys Tyr Gln Pro Gln Asp
   165                               170                               175

Thr Lys Leu His Ala Phe Ile Ser Ala Ala Leu Phe Gly Asp Ala Val
   180                               185                               190

Ser Ala Cys Val Met Arg Ala Asp Asp Lys Ala Pro Gly Phe Lys Ile
   195                               200                               205

Ala Lys Thr Gly Ser Tyr Phe Leu Pro Asp Ser Glu His Tyr Ile Lys
   210                               215                               220

Tyr Asp Val Lys Asp Ser Gly Phe His Phe Thr Leu Asp Lys Ala Val
  225                               230                               235                               240

Met Asn Ser Ile Lys Asp Val Ala Pro Met Met Glu Glu Leu Asn Phe
   245                               250                               255

Glu Thr Phe Asn Gln His Cys Ala Gln Asn Asp Phe Phe Ile Phe His
   260                               265                               270

Thr Gly Gly Arg Lys Ile Leu Asp Glu Leu Val Leu Gln Leu Asp Leu
   275                               280                               285

Glu Pro Gly Arg Val Ala Gln Ser Arg Asp Ser Leu Ser Glu Ala Gly
   290                               295                               300

Asn Ile Ala Ser Val Val Val Phe Asp Val Leu Lys Arg Gln Phe Asp
  305                               310                               315                               320

Ser Gly Pro Ala Asn Gly Ala Thr Gly Met Leu Ala Ala Phe Gly Pro
   325                               330                               335

Gly Phe Thr Ala Glu Met Ala Val Gly Lys Trp Val Ala
   340                               345

```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1269 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: complement (1..1269)
- (D) OTHER INFORMATION: /note= "phLE DNA sequence. SEQ ID NO:14 is translation (protein) of SEQ ID NO:13."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

CTTATCCTCC AGCGTCAAGC GAGCGGGCAG GGGGCCGTAG TCCGCACGGT CATTGAAAAC      60
AGAGCTGGCT TCCTTGAGCC GCAGGGACAG AAACCCGCCC ATCAAACACTAC CAGCCAGTGC      120
CAGAAACAGA ATGGCGGTAA GCCCCAGTT CACGGCGATG TACCCGGCGA CCACGGGCGC      180
AACGCCACCG CCCAGGATTT CTCCGCAACC CACCACCAGG CCCGTAGCGG TGGCCAGTAA      240
ACTGGGTGGC ACTGATTCGC TGGTCAGTGG GCCGACGGTG ATGCAGATCA GGCTGAAATT      300
GATGAAAGAT AAAAAGAACA GCTGGAGGAA CAGTAGCCAC GGTAACGGCG GGGAAATGAT      360
GAGCAAGCCG ACCAGTAGTG TGCTGATCAG GAAGCAGATG GAAACGACAG GCTTGC GGCC      420
CAGTTGGTCA GACAAACCGG GAATGACGAG CTGGCCGAAA AAACCACCCA GGCCGATCGC      480
GGAGATGATC ATGGCCATGG AGAAATTGCT CAGGTGCAAG ACGTCTGTCA GGTAGCTGGG      540
GAGCAGGGCG CACAGGACGA ATTGGCACGT CAGTATGCAT AGCATCAAGG CAATGTTGAG      600
GCGCACGTTG CCGCTGGACA GGGCTGTTCTG CCATTGGCTG CCGGAGGGTT CTACGAGCGG      660
CCTTGGATGG GCGGCCTGGC TCGGTTGGTA GGTTCGATAC AGATACCAGG CCACCAGCAG      720
GCCCCGCAAC GAGATGATGG CGAACACGGC GCGCCACGAT CCGAACATTT CAAACAATAC      780
GCCCCGCCAGC AGCGGCCCCA GGCACAGGCC GATGATGGGA AACAGTGCCT GCTGGATGCC      840
CAGGTTGAGC CCGCGTCGGC ACGGCTGCGA AACTTCATCG GTGACAATGA TGCTGACCGG      900
GGTGAAGGCG CCTTCGCAGA TCCCCATCAA GGCGCGCAGG AGCACCAGGC CCATAAGGCT      960
TGAGATCAAC GCAGATGCGC CGGCCAGGAG CGATACCAAG GTAATCGAAA GCACCAGCAG     1020
TTGCTTGGTG CCCAATCGCC TGATAGCAAC GCCCATGAAG AGGGCCGAGC CTCCCCAGGC     1080
AAATGCCAGG ATCGCCGATA ACAGGCCCCAG GTCCTGATAG TCCAGGGCCA GGTCATGCAT     1140
GATCACCGGG AACAACGGCA TGATAATGAA TCGATCAAGT CCTACCAGCC CGAAGCTCAG     1200
CGACAAAAGA ACGACCATGC GTCTTTCGTA GCCACCCCAA GGTCGAGTGG CAAGATACGT     1260
ACTCTCCAT                                     1269

```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 423 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```

Met Glu Ser Thr Tyr Leu Ala Thr Arg Pro Trp Gly Gly Tyr Glu Arg
 1           5           10           15
Arg Met Val Val Leu Leu Ser Leu Ser Phe Gly Leu Val Gly Leu Asp
          20           25           30
Arg Phe Ile Ile Met Pro Leu Phe Pro Val Ile Met His Asp Leu Ala
          35           40           45
Leu Asp Tyr Gln Asp Leu Gly Leu Leu Ser Ala Ile Leu Ala Phe Ala
 50           55           60
Trp Gly Gly Ser Ala Leu Phe Met Gly Val Ala Ile Arg Arg Leu Gly
 65           70           75           80
Thr Lys Gln Leu Leu Val Leu Ser Ile Thr Leu Val Ser Leu Leu Ala
          85           90           95
Gly Ala Ser Ala Leu Ile Ser Ser Leu Met Gly Leu Val Leu Leu Arg
          100          105          110
Ala Leu Met Gly Ile Cys Glu Gly Ala Phe Thr Pro Val Ser Ile Ile
          115          120          125
Val Thr Asp Glu Val Ser Gln Pro Cys Arg Arg Gly Leu Asn Leu Gly
          130          135          140
Ile Gln Gln Ala Leu Phe Pro Ile Ile Gly Leu Cys Leu Gly Pro Leu
145          150          155          160
Leu Ala Gly Val Leu Phe Glu Met Phe Gly Ser Trp Arg Ala Val Phe
          165          170          175
Ala Ile Ile Ser Leu Pro Gly Leu Leu Val Ala Trp Tyr Leu Tyr Arg
          180          185          190
Thr Tyr Gln Pro Ser Gln Ala Pro His Pro Arg Pro Leu Val Glu Pro
          195          200          205
Ser Gly Ser Gln Trp Arg Thr Ala Leu Ser Ser Gly Asn Val Arg Leu
210          215          220
Asn Ile Ala Leu Met Leu Cys Ile Leu Thr Cys Gln Phe Val Leu Cys
225          230          235          240
Ala Leu Leu Pro Ser Tyr Leu Thr Asp Val Leu His Leu Ser Asn Phe
          245          250          255
Ser Met Ala Met Ile Ile Ser Ala Ile Gly Leu Gly Gly Phe Phe Gly
          260          265          270
Gln Leu Val Ile Pro Gly Leu Ser Asp Gln Leu Gly Arg Lys Pro Val
          275          280          285
Val Ser Ile Cys Phe Leu Ile Ser Thr Leu Leu Val Gly Leu Leu Ile
290          295          300
Ile Ser Pro Pro Leu Pro Trp Leu Leu Phe Leu Gln Leu Phe Phe Leu
305          310          315          320

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Ser Phe Ile Asn Phe Ser Leu Ile Cys Ile Thr Val Gly Pro Leu Thr
          325                      330                      335

Ser Glu Ser Val Pro Pro Ser Leu Leu Ala Thr Ala Thr Gly Leu Val
          340                      345                      350

Val Gly Cys Gly Glu Ile Leu Gly Gly Gly Val Ala Pro Val Val Ala
          355                      360                      365

Gly Tyr Ile Ala Val Asn Trp Gly Leu Thr Ala Ile Leu Phe Leu Ala
          370                      375                      380

Leu Ala Gly Ser Leu Met Gly Gly Phe Leu Ser Leu Arg Leu Lys Glu
          385                      390                      395                      400

Ala Ser Ser Val Phe Asn Asp Arg Ala Asp Tyr Gly Pro Leu Pro Ala
          405                      410                      415

Arg Leu Thr Leu Glu Asp Lys
          420

```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 606 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..606
- (D) OTHER INFORMATION: /note= "phlF DNA sequence. SEQ ID NO:16 is translation (protein) of SEQ ID NO:15."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATG GCC CGT AAA CCG TCT CGG AGC TCC ATT GGC TCA TTG AGG AGC CCA	48
Met Ala Arg Lys Pro Ser Arg Ser Ser Ile Gly Ser Leu Arg Ser Pro	
1 5 10 15	
CAT ACG CAC AAA GCG ATC ATC ATC TCC GCT ATA GAA ACA CTC AAG GAG	96
His Thr His Lys Ala Ile Ile Ile Ser Ala Ile Glu Thr Leu Lys Glu	
20 25 30	
TGC GGT TAT TCA GGG TTG AGT ATC GAG GCT GTG GCT CGC CGT GCC GGC	144
Cys Gly Tyr Ser Gly Leu Ser Ile Glu Ala Val Ala Arg Arg Ala Gly	
35 40 45	
GCG AGC AAG CCG ACC ATC TAT CGA TGG TGG GGT AAC AAG GCG GCT TTG	192
Ala Ser Lys Pro Thr Ile Tyr Arg Trp Trp Gly Asn Lys Ala Ala Leu	
50 55 60	
ATC GCC GAA GTC TAC GAG AGC GAA AGC GAG CAG ATT CGC AAG GAG CCT	240
Ile Ala Glu Val Tyr Glu Ser Glu Ser Glu Gln Ile Arg Lys Glu Pro	
65 70 75 80	
GAT AAA GGA TCC TTC AAG GAG AAC CTC AAT TTC CTG CTG CTC AAT CTG	288
Asp Lys Gly Ser Phe Lys Glu Asn Leu Asn Phe Leu Leu Leu Asn Leu	
85 90 95	
TGG AAG GTC TGG AGA GAA ACG ATT TGC GGG GAG GCG TTT CGG TGT GTC	336
Trp Lys Val Trp Arg Glu Thr Ile Cys Gly Glu Ala Phe Arg Cys Val	
100 105 110	
ATC GCT GAA GCC CAG CTC GAC CCC AGT ACG CTG CCC AAG CTG AAG GAT	384
Ile Ala Glu Ala Gln Leu Asp Pro Ser Thr Leu Pro Lys Leu Lys Asp	
115 120 125	
GAA TTC ATG GAG CGT CGT CGG GAA TTG CCG CGA AAG CTG GTG GAA AAC	432
Glu Phe Met Glu Arg Arg Arg Glu Leu Pro Arg Lys Leu Val Glu Asn	
130 135 140	
GCC ATC CAG CAA GGT GAG TTG CCC AAG GAC ACG TCC CGT GAG TTG TTG	480
Ala Ile Gln Gln Gly Glu Leu Pro Lys Asp Thr Ser Arg Glu Leu Leu	
145 150 155 160	
TTG GAC ATG ATC TTC GGA TTT TGC TGG TAC AGG CTG TTG ACT GAG CAA	528
Leu Asp Met Ile Phe Gly Phe Cys Trp Tyr Arg Leu Leu Thr Glu Gln	
165 170 175	
CTG GAA GTG GAG GGT GAC ATC AAT GAA TTC ACG ACG CTT CTG TTG AAC	576
Leu Glu Val Glu Gly Asp Ile Asn Glu Phe Thr Thr Leu Leu Leu Asn	
180 185 190	
GGC GTG TTG CGT ACG ACT TCG GCG GCG GAG	606
Gly Val Leu Arg Thr Thr Ser Ala Ala Glu	
195 200	

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 202 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

Met Ala Arg Lys Pro Ser Arg Ser Ser Ile Gly Ser Leu Arg Ser Pro
 1              5              10              15
His Thr His Lys Ala Ile Ile Ile Ser Ala Ile Glu Thr Leu Lys Glu
          20              25              30
Cys Gly Tyr Ser Gly Leu Ser Ile Glu Ala Val Ala Arg Arg Ala Gly
          35              40              45
Ala Ser Lys Pro Thr Ile Tyr Arg Trp Trp Gly Asn Lys Ala Ala Leu
          50              55              60
Ile Ala Glu Val Tyr Glu Ser Glu Ser Glu Gln Ile Arg Lys Glu Pro
        65              70              75              80
Asp Lys Gly Ser Phe Lys Glu Asn Leu Asn Phe Leu Leu Leu Asn Leu
          85              90              95
Trp Lys Val Trp Arg Glu Thr Ile Cys Gly Glu Ala Phe Arg Cys Val
          100              105              110
Ile Ala Glu Ala Gln Leu Asp Pro Ser Thr Leu Pro Lys Leu Lys Asp
          115              120              125
Glu Phe Met Glu Arg Arg Arg Glu Leu Pro Arg Lys Leu Val Glu Asn
          130              135              140
Ala Ile Gln Gln Gly Glu Leu Pro Lys Asp Thr Ser Arg Glu Leu Leu
          145              150              155              160
Leu Asp Met Ile Phe Gly Phe Cys Trp Tyr Arg Leu Leu Thr Glu Gln
          165              170              175
Leu Glu Val Glu Gly Asp Ile Asn Glu Phe Thr Thr Leu Leu Leu Asn
          180              185              190
Gly Val Leu Arg Thr Thr Ser Ala Ala Glu
          195              200

```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1254 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1254

(D) OTHER INFORMATION: /note= "phlR DNA sequence. SEQ ID NO:18 is translation (protein) of SEQ ID NO:17."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATG GGA TAC GCC GGT ATA GGC ACA ACC ACG GAC GAA TAC CGG TTG GGT	48
Met Gly Tyr Ala Gly Ile Gly Thr Thr Thr Asp Glu Tyr Arg Leu Gly	
1 5 10 15	
CGT GTA GCG GTG CGC CAG GTG TTC GGC GAC CAG GAT GGC GCA ACC GCT	96
Arg Val Ala Val Arg Gln Val Phe Gly Asp Gln Asp Gly Ala Thr Ala	
20 25 30	
GGC TTC ACC CCA GGC CAG CAT CGA GCC ACA TGC TTC GCT GTT CTT GAG	144
Gly Phe Thr Pro Gly Gln His Arg Ala Thr Cys Phe Ala Val Leu Glu	
35 40 45	
GGT TTC AAG GGA TGG CAC CGG CAC GCC ATA GCG GGT TGC CGT GGG CGT	192
Gly Phe Lys Gly Trp His Arg His Ala Ile Ala Gly Cys Arg Gly Arg	
50 55 60	
GTT GTG CGC ATA GAT GCG CAT TTG CCG ACC AAA CGT TGC CAG GAC ATC	240
Val Val Arg Ile Asp Ala His Leu Pro Thr Lys Arg Cys Gln Asp Ile	
65 70 75 80	
CGC TTC GCG TCC TGC ATA GCC AAA TTT TTC AAA ATA TTC GGC GGT TGC	288
Arg Phe Ala Ser Cys Ile Ala Lys Phe Phe Lys Ile Phe Gly Gly Cys	
85 90 95	
GAG GGC AAA GGC GTC GGT GTG CGA AAT GCC CAG GAA ATA ATC GTA CTC	336
Glu Gly Lys Gly Val Gly Val Arg Asn Ala Gln Glu Ile Ile Val Leu	
100 105 110	
ACA TTC GGT ACT GGA GCC GAT GTA TTC GGC ATA GTT GAA GTG GTC GGT	384
Thr Phe Gly Thr Gly Ala Asp Val Phe Gly Ile Val Glu Val Val Gly	
115 120 125	
CAT TTT TTC AAA GCC ACC ACA CAG GAC GAT GTC GTA CTC ACC CGA GGC	432
His Phe Phe Lys Ala Thr Thr Gln Asp Asp Val Val Leu Thr Arg Gly	
130 135 140	
GAC CAT CTG ATG GGC CAT CTG AAA GGA AAC CGA GCT GCT GGT GCA GTT	480
Asp His Leu Met Gly His Leu Lys Gly Asn Arg Ala Ala Gly Ala Val	
145 150 155 160	
GGC AGT GCT CAT GAA CGT CGG GGC AGG GCT GAT GCC CAG GGC ATC GGA	528
Gly Ser Ala His Glu Arg Arg Gly Arg Ala Asp Ala Gln Gly Ile Gly	
165 170 175	
AAT AGT CGG GCC CAG GCC GCC GTA TTC GGA AAT ACC TTC ACC GTG ATA	576
Asn Ser Arg Ala Gln Ala Ala Val Phe Gly Asn Thr Phe Thr Val Ile	
180 185 190	
TCC ATA AGC GAC TGC CTG AAG TTC ACG GGG ATG CAT CTT GAT GGC GTT	624
Ser Ile Ser Asp Cys Leu Lys Phe Thr Gly Met His Leu Asp Gly Val	
195 200 205	

GAG CGC CTG ATA GGC GGA CTC GAC GAT CAT CTC CTT GAA GGT TTG ACG Glu Arg Leu Ile Gly Gly Leu Asp Asp His Leu Leu Glu Gly Leu Thr 210 215 220	672
GAC TCT GGA GCT GCC GGG TTT GGA AGT ATA GGC AGC CGA AAC GAT AGC Asp Ser Gly Ala Ala Gly Phe Gly Ser Ile Gly Ser Arg Asn Asp Ser 225 230 235 240	720
AAC GCG TCG TGC GCT CAT TGG AAG TGC TCC TTG CTG GAT GGT TGG GAA Asn Ala Ser Cys Ala His Trp Lys Cys Ser Leu Leu Asp Gly Trp Glu 245 250 255	768
TCA GAG GTA GGC TGT CAG GGC GTA GTC AGG CCG CAA GTA TTT GAA CTC Ser Glu Val Gly Cys Gln Gly Val Val Arg Pro Gln Val Phe Glu Leu 260 265 270	816
GTA CTT GAT CGA CGT CCC GTA ATC CAC GTA ATA CTT GTC TTC CAG CAG Val Leu Asp Arg Arg Pro Val Ile His Val Ile Leu Val Phe Gln Gln 275 280 285	864
CGT GCG CAG CGC AAC GTT GGT CTT TTG GTA GGC TTC GAT GGC ATC GGT Arg Ala Gln Arg Asn Val Gly Leu Leu Val Gly Phe Asp Gly Ile Gly 290 295 300	912
CAC TGT CAA CGC AAT CGC ATC GCT GCC CGC ACC AAA CCC GTA CGA CAC His Cys Gln Arg Asn Arg Ile Ala Ala Arg Thr Lys Pro Val Arg His 305 310 315 320	960
CAA GAG GAT TTT TTC ACC CGG ACG CGC TCG GTC CAG TAC GCT CAC CAA Gln Glu Asp Phe Phe Thr Arg Thr Arg Ser Val Gln Tyr Ala His Gln 325 330 335	1008
GCC CAG CAA CGG ACT CGC GGG CCC CGC ATC ACC GAC ACT CTG GGC ATA Ala Gln Gln Arg Thr Arg Gly Pro Arg Ile Thr Asp Thr Leu Gly Ile 340 345 350	1056
AAT GCC AGG TTC GAT CTG CGC TTT GGT GAA GCC CAG GCC TTT GCC AAG Asn Ala Arg Phe Asp Leu Arg Phe Gly Glu Ala Gln Ala Phe Ala Lys 355 360 365	1104
AGA GAA GGG GGT CGA AAC CAG GTT TTG CTG GAA TAC GAC ATA GTC GAA Arg Glu Gly Gly Arg Asn Gln Val Leu Leu Glu Tyr Asp Ile Val Glu 370 375 380	1152
ATC GCT GGC CTG TAC ATT CAT CTT GGC CAT CAA TCC CGA CGC AGC ACG Ile Ala Gly Leu Tyr Ile His Leu Gly His Gln Ser Arg Arg Ser Thr 385 390 395 400	1200
ATG GGT CTG GTC TTC AAG GCC AAT GCT GTT CTT GTC GGA GCC CAG CCC Met Gly Leu Val Phe Lys Ala Asn Ala Val Leu Val Gly Ala Gln Pro 405 410 415	1248
CAT TCC His Ser	1254

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 418 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```

Met Gly Tyr Ala Gly Ile Gly Thr Thr Thr Asp Glu Tyr Arg Leu Gly
 1           5           10           15
Arg Val Ala Val Arg Gln Val Phe Gly Asp Gln Asp Gly Ala Thr Ala
          20           25           30
Gly Phe Thr Pro Gly Gln His Arg Ala Thr Cys Phe Ala Val Leu Glu
          35           40           45
Gly Phe Lys Gly Trp His Arg His Ala Ile Ala Gly Cys Arg Gly Arg
          50           55           60
Val Val Arg Ile Asp Ala His Leu Pro Thr Lys Arg Cys Gln Asp Ile
          65           70           75           80
Arg Phe Ala Ser Cys Ile Ala Lys Phe Phe Lys Ile Phe Gly Gly Cys
          85           90           95
Glu Gly Lys Gly Val Gly Val Arg Asn Ala Gln Glu Ile Ile Val Leu
          100          105          110
Thr Phe Gly Thr Gly Ala Asp Val Phe Gly Ile Val Glu Val Val Gly
          115          120          125
His Phe Phe Lys Ala Thr Thr Gln Asp Asp Val Val Leu Thr Arg Gly
          130          135          140
Asp His Leu Met Gly His Leu Lys Gly Asn Arg Ala Ala Gly Ala Val
          145          150          155          160
Gly Ser Ala His Glu Arg Arg Gly Arg Ala Asp Ala Gln Gly Ile Gly
          165          170          175
Asn Ser Arg Ala Gln Ala Ala Val Phe Gly Asn Thr Phe Thr Val Ile
          180          185          190
Ser Ile Ser Asp Cys Leu Lys Phe Thr Gly Met His Leu Asp Gly Val
          195          200          205
Glu Arg Leu Ile Gly Gly Leu Asp Asp His Leu Leu Glu Gly Leu Thr
          210          215          220

Asp Ser Gly Ala Ala Gly Phe Gly Ser Ile Gly Ser Arg Asn Asp Ser
          225          230          235          240
Asn Ala Ser Cys Ala His Trp Lys Cys Ser Leu Leu Asp Gly Trp Glu
          245          250          255
Ser Glu Val Gly Cys Gln Gly Val Val Arg Pro Gln Val Phe Glu Leu
          260          265          270
Val Leu Asp Arg Arg Pro Val Ile His Val Ile Leu Val Phe Gln Gln
          275          280          285

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Arg Ala Gln Arg Asn Val Gly Leu Leu Val Gly Phe Asp Gly Ile Gly
 290 295 300
 His Cys Gln Arg Asn Arg Ile Ala Ala Arg Thr Lys Pro Val Arg His
 305 310 315 320
 Gln Glu Asp Phe Phe Thr Arg Thr Arg Ser Val Gln Tyr Ala His Gln
 325 330 335
 Ala Gln Gln Arg Thr Arg Gly Pro Arg Ile Thr Asp Thr Leu Gly Ile
 340 345 350
 Asn Ala Arg Phe Asp Leu Arg Phe Gly Glu Ala Gln Ala Phe Ala Lys
 355 360 365
 Arg Glu Gly Gly Arg Asn Gln Val Leu Leu Glu Tyr Asp Ile Val Glu
 370 375 380
 Ile Ala Gly Leu Tyr Ile His Leu Gly His Gln Ser Arg Arg Ser Thr
 385 390 395 400
 Met Gly Leu Val Phe Lys Ala Asn Ala Val Leu Val Gly Ala Gln Pro
 405 410 415
 His Ser

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 390 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Pseudomonas fluorescens*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..390
- (D) OTHER INFORMATION: /note= "phlF, truncated, DNA sequence. SEQ ID NO:20 is translation (protein) of SEQ ID NO:19. "

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATG GCC CGT AAA CCG TCT CGG AGC TCC ATT GGC TCA TTG AGG AGC CCA	48
Met Ala Arg Lys Pro Ser Arg Ser Ser Ile Gly Ser Leu Arg Ser Pro	
1 5 10 15	
CAT ACG CAC AAA GCG ATC ATC ATC TCC GCT ATA GAA ACA CTC AAG GAG	96
His Thr His Lys Ala Ile Ile Ile Ser Ala Ile Glu Thr Leu Lys Glu	
20 25 30	

```

TGC GGT TAT TCA GGG TTG AGT ATC GAG GCT GTG GCT CGC CGT GCC GGC      144
Cys Gly Tyr Ser Gly Leu Ser Ile Glu Ala Val Ala Arg Arg Ala Gly
      35                      40                      45

GCG AGC AAG CCG ACC ATC TAT CGA TGG TGG GGT AAC AAG GCG GCT TTG      192
Ala Ser Lys Pro Thr Ile Tyr Arg Trp Trp Gly Asn Lys Ala Ala Leu
      50                      55                      60

ATC GCC GAA GTC TAC GAG AGC GAA AGC GAG CAG ATT CGC AAG GAG CCT      240
Ile Ala Glu Val Tyr Glu Ser Glu Ser Glu Gln Ile Arg Lys Glu Pro
      65                      70                      75                      80

GAT AAA GGA TCC TTC AAG GAG AAC CTC AAT TTC CTG CTG CTC AAT CTG      288
Asp Lys Gly Ser Phe Lys Glu Asn Leu Asn Phe Leu Leu Leu Asn Leu
      85                      90                      95

TGG AAG GTC TGG AGA GAA ACG ATT TGC GGG GAG GCG TTT CGG TGT GTC      336
Trp Lys Val Trp Arg Glu Thr Ile Cys Gly Glu Ala Phe Arg Cys Val
      100                      105                      110

ATC GCT GAA GCC CAG CTC GAC CCC AGT ACG CTG CCC AAG CTG AAG GAT      384
Ile Ala Glu Ala Gln Leu Asp Pro Ser Thr Leu Pro Lys Leu Lys Asp
      115                      120                      125

GAA TTC      390
Glu Phe
      130

```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 130 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```

Met Ala Arg Lys Pro Ser Arg Ser Ser Ile Gly Ser Leu Arg Ser Pro
 1              5              10              15

His Thr His Lys Ala Ile Ile Ile Ser Ala Ile Glu Thr Leu Lys Glu
      20              25              30

Cys Gly Tyr Ser Gly Leu Ser Ile Glu Ala Val Ala Arg Arg Ala Gly
      35              40              45

Ala Ser Lys Pro Thr Ile Tyr Arg Trp Trp Gly Asn Lys Ala Ala Leu
      50              55              60

Ile Ala Glu Val Tyr Glu Ser Glu Ser Glu Gln Ile Arg Lys Glu Pro
      65              70              75              80

Asp Lys Gly Ser Phe Lys Glu Asn Leu Asn Phe Leu Leu Leu Asn Leu
      85              90              95

Trp Lys Val Trp Arg Glu Thr Ile Cys Gly Glu Ala Phe Arg Cys Val
      100              105              110

```

Ile Ala Glu Ala Gln Leu Asp Pro Ser Thr Leu Pro Lys Leu Lys Asp
115 120 125

Glu Phe
130

Applicant's or agent's file reference number	700/320 (PCT)	International application
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page (see cont. sheet) . line (see cont. sheet)	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Agricultural Research Service Culture Collection (NRRL)	
Address of depositary institution (including postal code and country) 1815 N. University Street Peoria, Illinois 61604 United States of America	
Date of deposit (see continuation sheet)	Accession Number (see continuation sheet)
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
3 microorganisms are disclosed in the specification and listed on continuation sheet. In respect to those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on
Authorized officer R. Hill	Authorized officer

Application: PCT/US96 - NEW
Filed: June 26, 1996
Attorney Ref.: A700/320

2/2

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(continuation sheet)

The following microorganisms, for which Rule 28(4) EPC treatment is requested, were deposited under the terms of the Budapest Treaty in the USDA, Agricultural Research Service Culture Collection in Peoria, Illinois:

<u>Date of Deposit</u>	<u>Accession Number</u>	<u>Specification</u>
June 21, 1995	NRRL B-21477	page 15, lines 22-27
June 21, 1995	NRRL B-21476	page 16, lines 1-6
June 21, 1995	NRRL B-21474	page 39, lines 4-7

CLAIMS

1. An isolated and purified DNA sequence which functions specifically in the biosynthesis of 2,4-diacetylphloroglucinol (Phl).
2. The DNA sequence of claim 1, wherein said DNA sequence contains one or more *phl* genes or fragment thereof.
3. The DNA sequence of claim 1, wherein said DNA sequence is genomic DNA.
4. The DNA sequence of claim 1, wherein said DNA sequence is selected from a group consisting of *phlA*, *phlB*, *phlC*, *phlD*, *phlE*, *phlF*, and *phlR* genes, or combinations thereof, which genes function in the production of Phl.
5. The DNA sequence of claim 4, wherein said DNA sequence is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4.
6. The DNA sequence of claim 4, wherein said DNA sequence is selected from the group consisting of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, and SEQ ID NO:19 or combinations thereof.
7. The DNA sequence of claim 1, wherein said sequence encodes a *phl* gene protein.
8. The DNA sequence of claim 7, wherein said *phl* gene protein is selected from the group consisting of PhlA, PhlB, PhlC, PhlD, PhlE, PhlF, and PhlR, or combinations thereof.
9. The DNA sequence of claim 1, where said sequence consists of the *SalI-EcoRI* fragment of pPHL5122.
10. The DNA sequence of claim 1, where said sequence consists of the *SalI-BamHI* fragment of pPHL5120.
11. A recombinant DNA sequence wherein said DNA sequence of claim 1 is operably linked to a regulatory sequence.
12. A transgenic bacterial strain into which the recombinant DNA sequence of claim 11 has been introduced, said strain having the conferred or enhanced ability to produce Phl.
13. A protein encoded by a DNA sequence which functions specifically in the biosynthesis of Phl.
14. The protein of claim 13, wherein said protein is selected from the group consisting of PhlA, PhlB, PhlC, PhlD, PhlE, PhlF, and PhlR, or combinations thereof.

15. A method for introducing or enhancing the expression of Phl in a host strain, comprising transforming said strain with a DNA sequence of claim 1.

16. A method for detecting a DNA sequence which functions specifically in the biosynthesis of Phl, comprising contacting the DNA sequence of claim 1 or portion thereof with a preparation of genomic DNA from a microorganism strain under hybridization conditions providing detection of DNA sequences having substantial sequence homology to said sequence.

17. A method for rendering a bacterial strain effective against fungal pathogens, said method comprising introducing into the bacterial strain the DNA sequence of claim 1.

18. A method for producing Phl comprising introducing a sequence of claim 1 into the genome of a bacterium, culturing the transformed bacterium to produce Phl and extracting Phl from the culture.

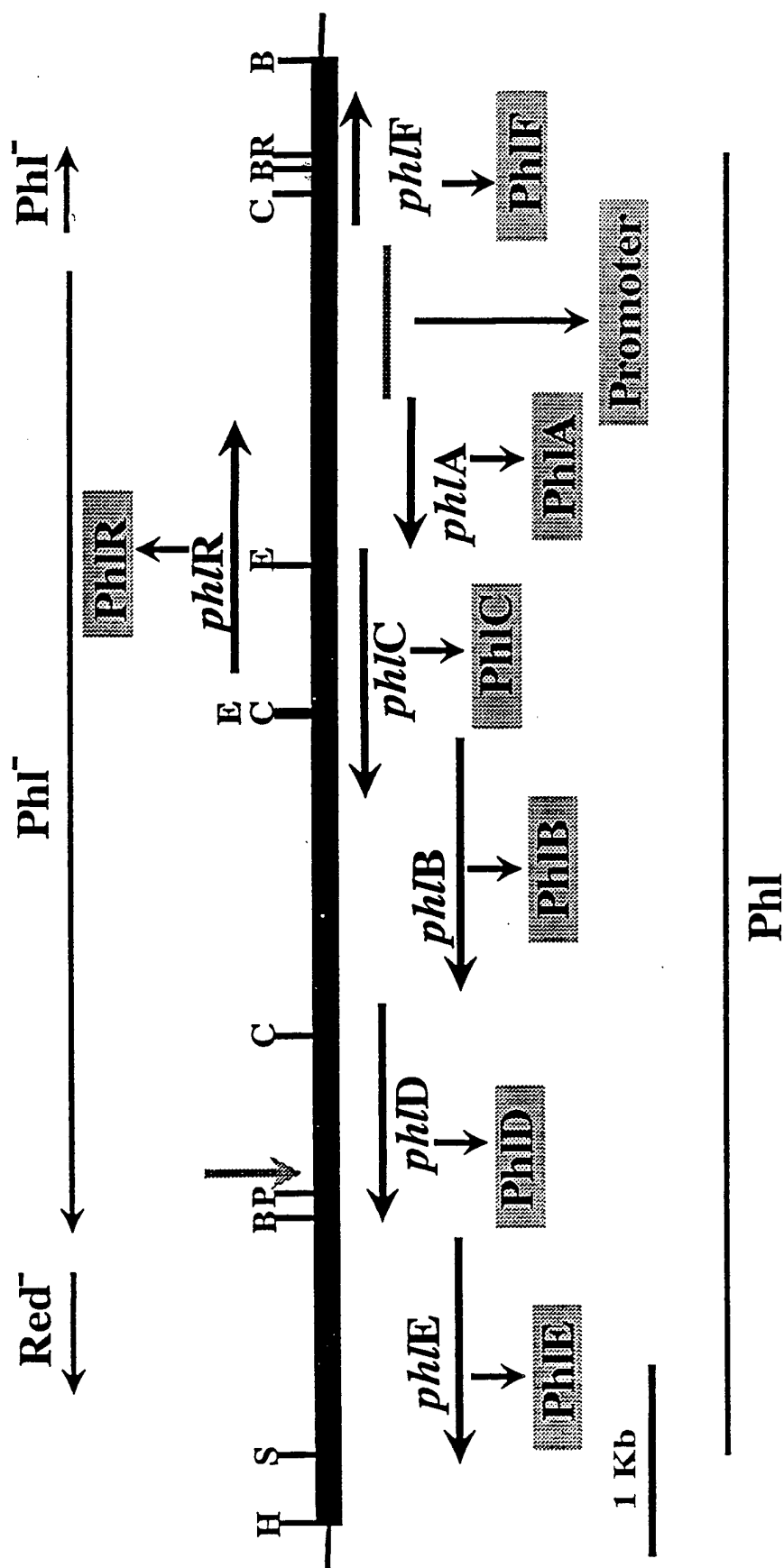


FIG. 1

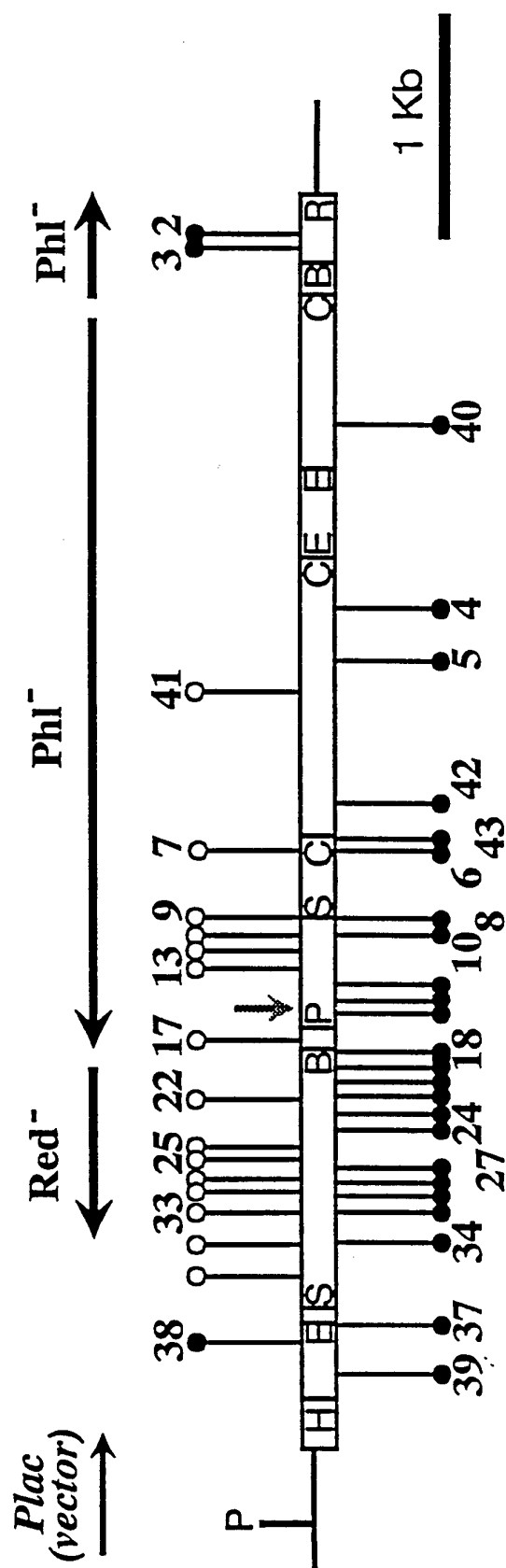


FIG. 2

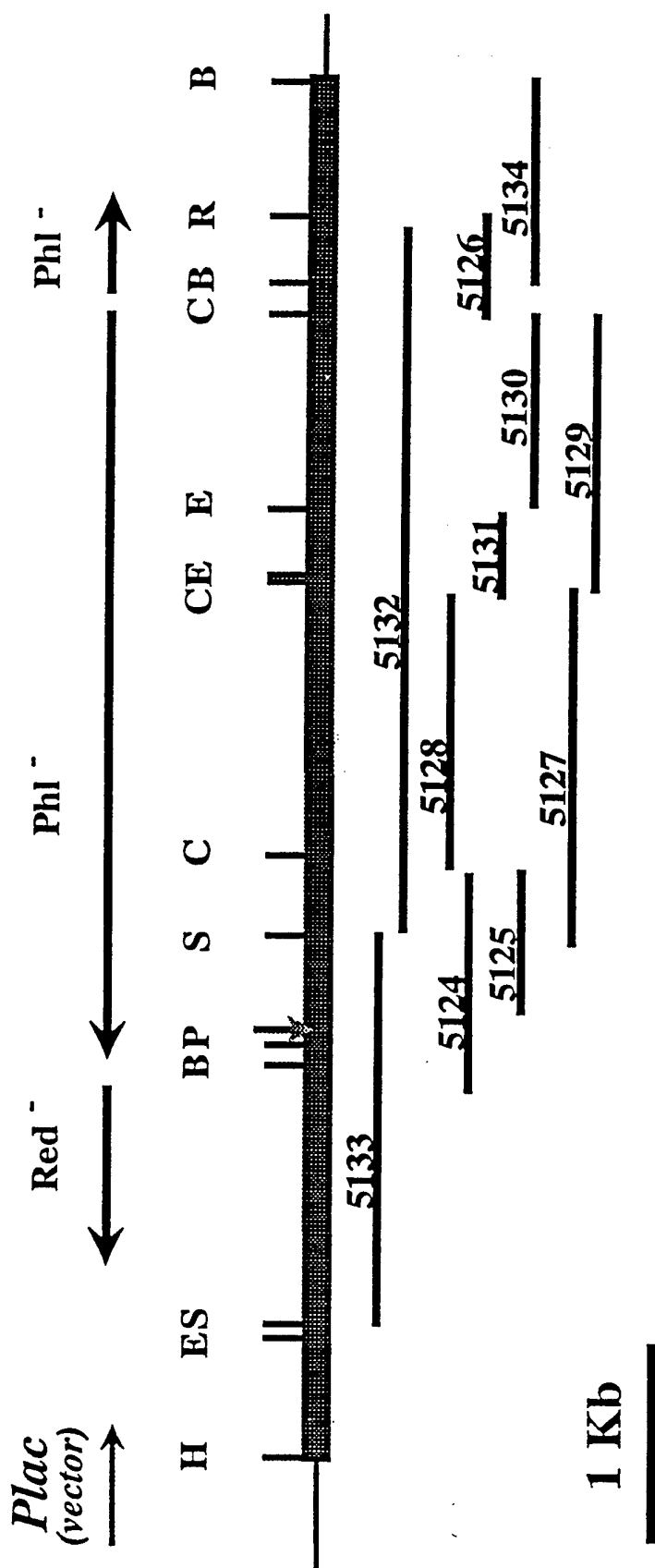


FIG. 3

PROPOSED BIOSYNTHETIC PATHWAY
2,4-DIACETYLPHLOROGLUCINOL

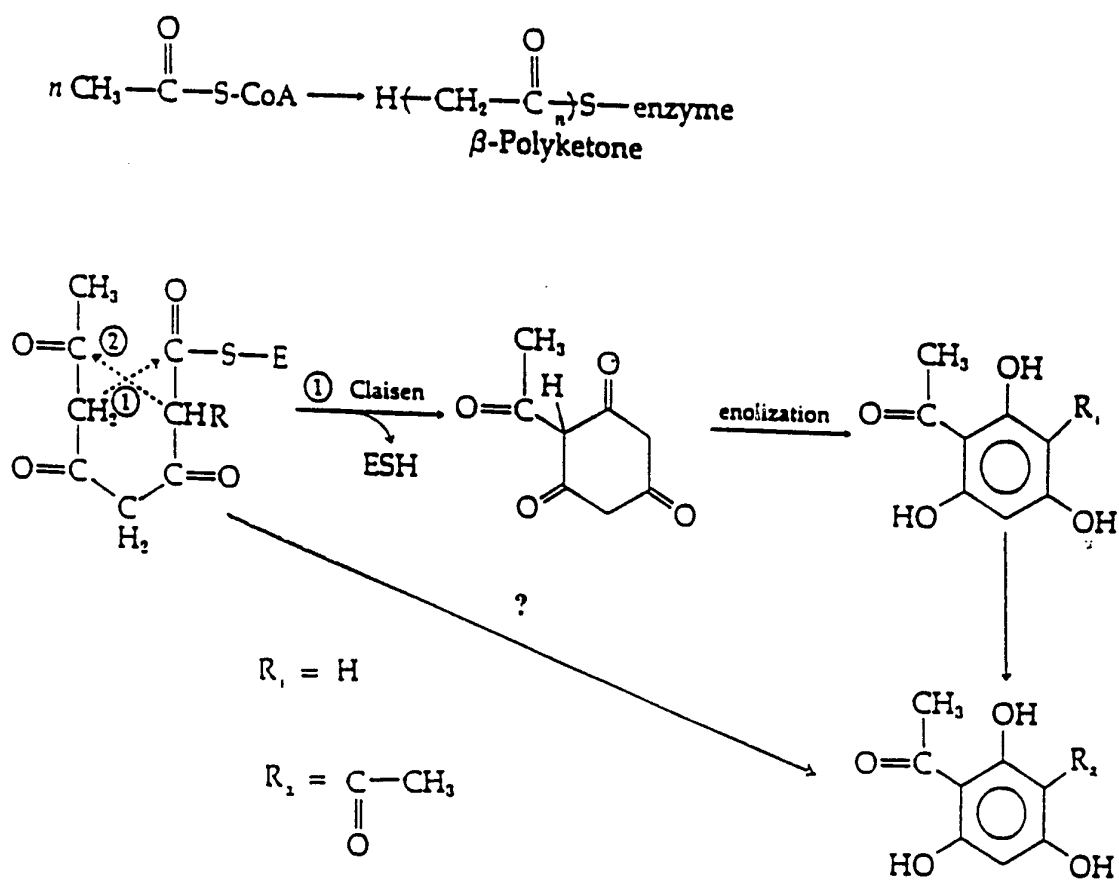


FIG. 4

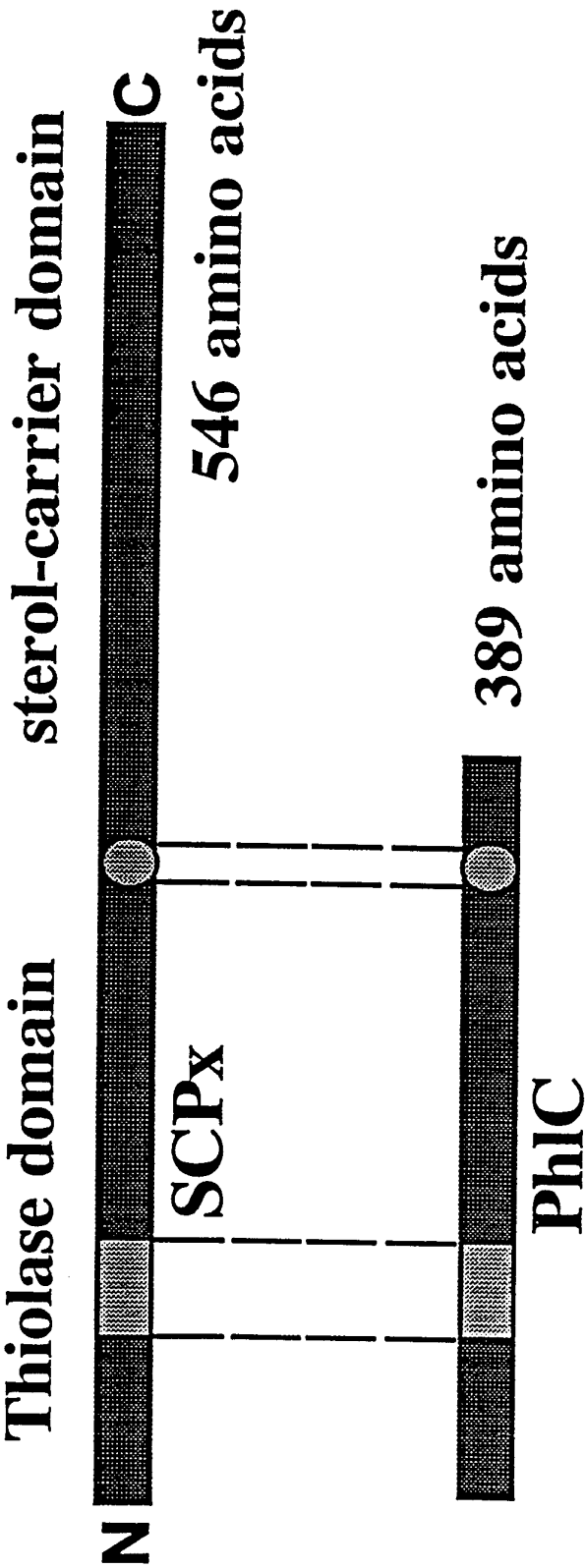


FIG. 5

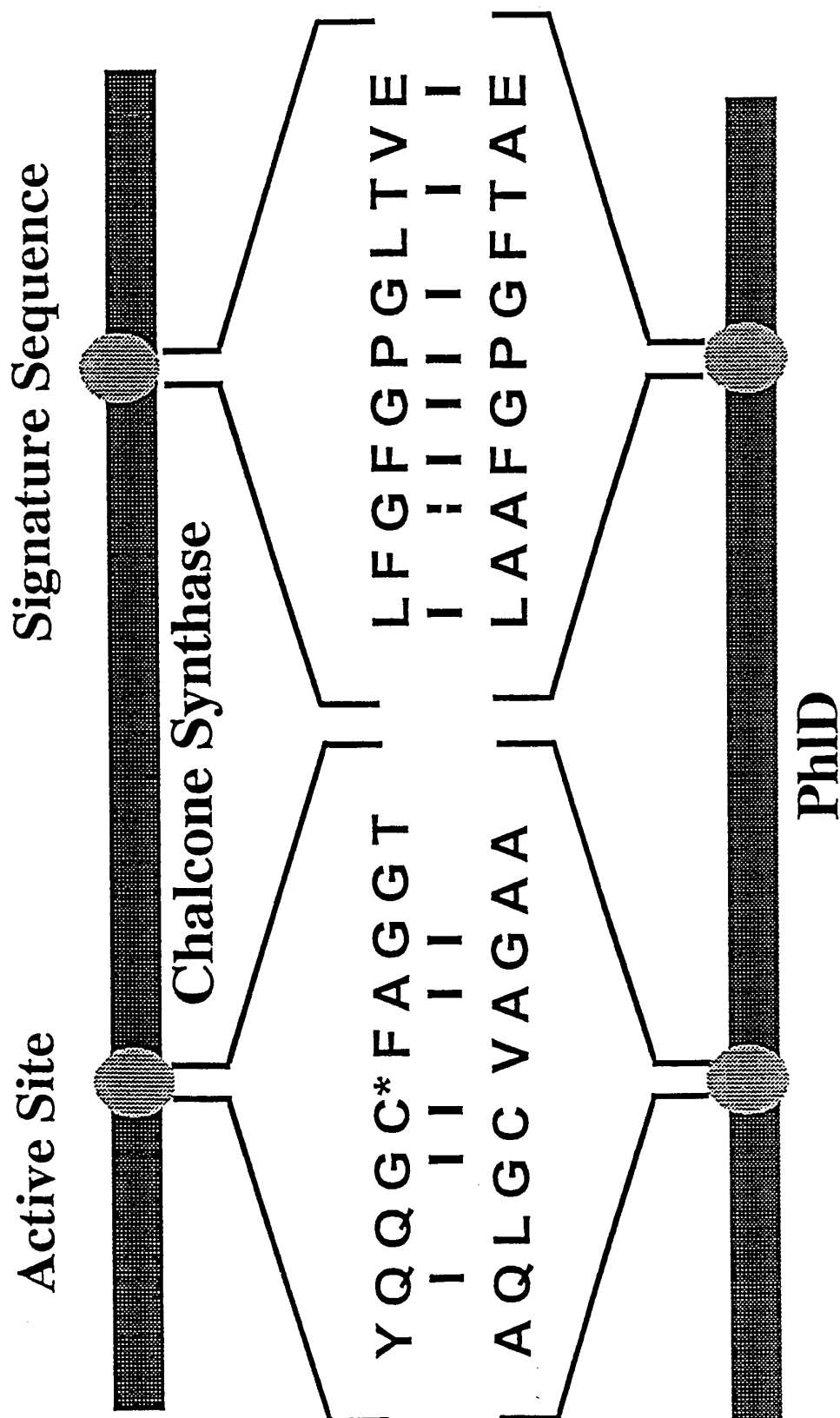


FIG. 6

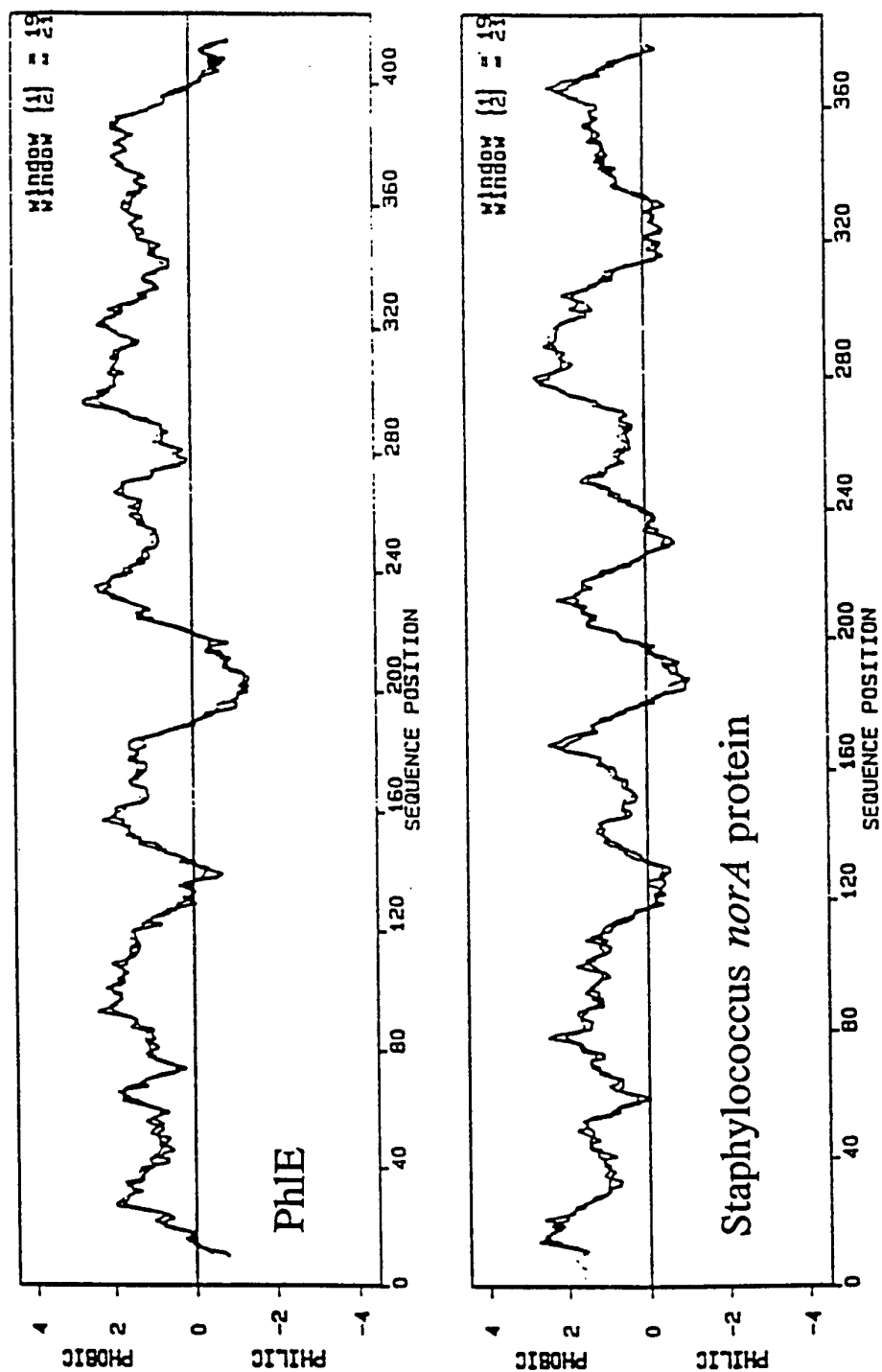


FIG. 7

Protein	Sequence	HTH Score
434 rep	Q A E L A Q K V G T T Q Q S I E Q I E N	1875
434 cro	Q T E L A T K A G V K Q Q S I Q L I E A	1274
Lamb rep	Q E S V A D K M G M G Q S G V G A I E N	1553
Lamb cro	Q T K T A K D L G V Y Q S A I N K A I H	1626
CRP	R Q E I G E I V G C S R E T V G R I L K	1916
Lac rep	L Y D V A R L A G V S Y Q T V S R V V N	2052
Ec 1721	T R K L A E R L G V Q Q P A L Y W H E R	2090
Ec tetc	T R R L A E R L G V Q Q P A L Y W H E K	1997
Tet rep	T R K L A Q K L G V E Q P T L Y W H V K	1994
PhlF	I E A V A R R A G A S K P T I Y R W W G	1886
Myco lep	I D E L A E R A G V S R A L M I H E P	1487
Ec bet	I A Q L A R R A G V S T G I I S H Y E R	2015
Consensus	Q E E L A E R L G V S Q P T I Q R I L K	

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10986

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 189, 195, 252.3, 320.1; 530/350; 536/22.1, 23.1, 23.2, 23.7, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CA

search terms: phl, pseudomonas, fluorescens, diacetylphloroglucinol

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BANGERA, et al. "Genetic analysis of the 2,4-diacetylphloroglucinol biosynthetic locus from Pseudomonas fluorescens Q2-87", Curr. Plant, Sci. Biotechnol. Agric. (Advances in Molecular Genetics of Plant-Microbe Interactions). 1994, Abstract, see entire document.	1-18
A	MAURHOFER, et al. "Influence of plant species on disease suppression by Pseudomonas fluorescens strain CHAO with enhanced antibiotic production", Plant Pathology, February 1995, Vol. 44, No. 1, pages 40-50, see entire document.	1-18

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

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07 NOV 1996

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10986

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SHANAHAN et al., "Isolation of 2,4-Diacetylphloroglucinol from a Fluorescent Pseudomonad and Investigation of Physiological Parameters influencing Its Production", Applied and Environmental microbiology, January 1992, Vol. 58, No. 1, pages 353-358, see entire document.	1-18
A	FENTON et al., "Exploitation of Gene(s) Involved in 2,4-Diacetylphloroglucinol Biosynthesis To Confer a New Biocontrol Capability to a Pseudomonas Strain", Applied and Environmental Microbiology, December 1992, Vol. 58, No. 12, pages 3873-3878, see entire document.	1-18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/10986

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07H 19/00, 21/00, 21/02, 21/04; C07K 1/00, 14/00, 17/00; C12N 9/02, 9/14, 1/20, 15/00, 15/09, 15/63, 15/70, 15/74; C12P 21/06; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 69.1, 189, 195, 252.3, 320.1; 530/350; 536/22.1, 23.1, 23.2, 23.7, 24.1