METHODS OF DETECTING AND CONTROLLING MUCOID PSEUDOMONAS BIOFILM PRODUCTION

Inventors: Hongwei D. Yu, Huntington, WV (US); Dongru Qiu, Huntington, WV (US)

Assignee: Marshall University Research Corporation, Huntington, WV (US)

Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 176 days.

Prior Publication Data

Related U.S. Application Data
Continuation of application No. 11/730,186, filed on Mar. 29, 2007, now Pat. No. 7,781,166.

Provisional application No. 60/787,497, filed on Mar. 31, 2006.

References Cited
U.S. PATENT DOCUMENTS
6,083,691 A 7/2000 Deretic et al.
6,355,469 B1 3/2002 Lam
6,426,187 B1 7/2002 Deretic et al.
6,551,795 B1 4/2003 Rubenfield et al.
6,610,836 B1 8/2003 Breton et al.
6,777,223 B2 8/2004 Xu
6,830,745 B1 12/2004 Budny et al.

OTHER PUBLICATIONS


Primary Examiner — Brian J Gangle
Attorney, Agent, or Firm — Stites & Harbison PLLC; Terry L. Wright

Compositions and methods for detecting and controlling the conversion to mucoidy in Pseudomonas aeruginosa are disclosed. The present invention provides for detecting the switch from nonmucoid to mucoid state of P. aeruginosa by measuring mucE expression or MucE protein levels. The interaction between MucE and AlgW controls the switch to mucoidy in wild type P. aeruginosa. Also disclosed is an alginate biosynthesis heterologous expression system for use in screening candidate substances that inhibit conversion to mucoidy.

12 Claims, 16 Drawing Sheets
OTHER PUBLICATIONS


ATGGGTTTCCGGCCAGTTAGCAACGTTTGCAGCAATCAAACCTGCAGCCCGCTCGGC
AAGTTTTTCCTGCTTGCCCTGCTGGTCCTCGGCCTGGAATCGGTAAGCCATCCGCGCCCGGC
CCGGTCCAGGCCCCCTCCIAGCAAGCAGCCACCCTCCAGCCCTGCCTTGCTACTCCCG
CTCGGCCTCGACGGCCGCCCGCCCGCCAGGGCCGAGATGTGGAACGTCGGCTGTC
CGGCACCGTCAGCGTGCGTAGAAGTTTGCATGGGGGTGTGTTTTGA

FIGURE 1
MGFRPVSRDLMLQALGVFLCLLVLGLESVSPAGPVQAPSFSQGTASPSFATPLGL
DGPARAAEMWNVGLSGAVSVRDELRWVF

FIGURE 2
ATGGGGAACCTGCTCAGGAAAGGCCAGGTCGCGCTTGTCAGAATATTTCAACGGGC
TGATCCGGTGCGTCTTCTCAGTTTGAATGCTGCGGCTTTATCTGGGAATCAAGTCCTGT
ACCGTGCCACGCTCCACACGCAGCGGCTGCTGTCAGGCCCTCGGCCGCGCATAGGGCAATACCC
GGCGTCTGCCCTGGCAGCGCACTCAACCTGACCCTGGGAGCGGACGCCGAGCCCGT
GATCGGTACACGGCTACCCACGAACCTGGCAACGCCACCGGCCCAGCGCTGGGTGT
TCTAG

FIGURE 3
MGNLLRKQVALVRIFSGDDPVRLLSLMLAAYLGISACTVPASTAGCCQPSGIGQYPAS
ALPAGDSNLTLDAEPVIGRTALPTNLQPPAPRWWF

FIGURE 4
FIGURE 5A

# of insertions in the α-gal promoter region
**FIGURE 6**

**A**

VE1: PAO1-(algUmucABC)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4h</td>
<td>*</td>
</tr>
<tr>
<td>8h</td>
<td>*</td>
</tr>
<tr>
<td>12h</td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>*</td>
</tr>
<tr>
<td>48h</td>
<td>*</td>
</tr>
<tr>
<td>72h</td>
<td>*</td>
</tr>
</tbody>
</table>

**AlgU**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4h</td>
<td>2.9</td>
</tr>
<tr>
<td>8h</td>
<td>3.3</td>
</tr>
<tr>
<td>12h</td>
<td>2.5</td>
</tr>
<tr>
<td>24h</td>
<td>4.0</td>
</tr>
<tr>
<td>48h</td>
<td>3.6</td>
</tr>
<tr>
<td>72h</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**MucB**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4h</td>
<td>3.1</td>
</tr>
<tr>
<td>8h</td>
<td>2.3</td>
</tr>
<tr>
<td>12h</td>
<td>2.0</td>
</tr>
<tr>
<td>24h</td>
<td>2.9</td>
</tr>
<tr>
<td>48h</td>
<td>3.4</td>
</tr>
<tr>
<td>72h</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**B**

PAO1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4h</td>
<td></td>
</tr>
<tr>
<td>8h</td>
<td></td>
</tr>
<tr>
<td>12h</td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td></td>
</tr>
<tr>
<td>48h</td>
<td></td>
</tr>
<tr>
<td>72h</td>
<td></td>
</tr>
</tbody>
</table>

**AlgU**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4h</td>
<td>1.0</td>
</tr>
<tr>
<td>8h</td>
<td>1.0</td>
</tr>
<tr>
<td>12h</td>
<td>1.0</td>
</tr>
<tr>
<td>24h</td>
<td>1.0</td>
</tr>
<tr>
<td>48h</td>
<td>1.0</td>
</tr>
<tr>
<td>72h</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**MucB**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4h</td>
<td>1.0</td>
</tr>
<tr>
<td>8h</td>
<td>1.0</td>
</tr>
<tr>
<td>12h</td>
<td>1.0</td>
</tr>
<tr>
<td>24h</td>
<td>1.0</td>
</tr>
<tr>
<td>48h</td>
<td>1.0</td>
</tr>
<tr>
<td>72h</td>
<td>1.0</td>
</tr>
</tbody>
</table>
**FIGURE 7**

**A**

VE2: PAO1-mucE<sup>+</sup>

<table>
<thead>
<tr>
<th>Alginate</th>
<th>4h</th>
<th>8h</th>
<th>12h</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- AlgU

<table>
<thead>
<tr>
<th></th>
<th>4.7</th>
<th>3.4</th>
<th>2.4</th>
<th>2.1</th>
<th>1.9</th>
<th>1.5</th>
</tr>
</thead>
</table>

- MucB

<table>
<thead>
<tr>
<th></th>
<th>2.6</th>
<th>1.7</th>
<th>1.5</th>
<th>1.6</th>
<th>1.9</th>
<th>1.7</th>
</tr>
</thead>
</table>

**B**

<table>
<thead>
<tr>
<th>bps</th>
<th>PAO1</th>
<th>VE2</th>
<th>VE3</th>
<th>PAO1</th>
<th>VE2</th>
<th>VE3</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- AlgU

<table>
<thead>
<tr>
<th></th>
<th>1,000</th>
<th>900</th>
<th>800</th>
<th>700</th>
<th>600</th>
<th>500</th>
</tr>
</thead>
</table>

- MucE

<table>
<thead>
<tr>
<th></th>
<th>400</th>
<th>300</th>
<th>200</th>
<th>100</th>
</tr>
</thead>
</table>
FIGURE 8
FIGURE 9
FIGURE 13
FIGURE 15

Yellow: Peptidase_M50 domain
Blue: PDZ domain
Fink: overlap between peptidase and PDZ domain
Methods of Detecting and Controlling Mucoid Pseudomonas Biofilm Production

Cross Reference to Related Applications

The present application is a continuation of U.S. patent application Ser. No. 11/730,186, filed on Mar. 29, 2007, now U.S. Pat. No. 7,781,166 and claims the benefit of the filing date of U.S. Provisional Patent Application No. 60/787,497, filed Mar. 31, 2006, which is incorporated herein by reference in its entirety.

Statement regarding Federally-Sponsored Research and Development

Statement under MPEP 310. The U.S. government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of NNA04CC74G awarded by the National Aeronautics and Space Administration (NASA).

Part of the work performed during development of this invention utilized U.S. government funds. The U.S. government has certain rights in this invention.

Names of the Parties to a Joint Research Agreement

Not Applicable.

Background of the Invention

1. Field of the Invention

The present invention relates to the identification and use of positive regulators of alginate production in Pseudomonas aeruginosa. One aspect of the invention provides compositions and methods for the early detection and diagnosis of the conversion to mucoidy of Pseudomonas aeruginosa. The present invention also provides a molecular mechanism for detecting the conversion from the nonmucoid to the mucoid state, including molecular probes for the early detection of this disease state.

2. Background Art

Cystic Fibrosis (CF) is the most common inheritable lethal disease among Caucasians. The leading cause of high morbidity and mortality in CF patients are the chronic respiratory infections caused by Pseudomonas aeruginosa. Pseudomonas aeruginosa is an aerobic, motile, gram-negative bacterium with a simple metabolic demand that allows it to thrive in diverse environments. P. aeruginosa normally inhabits soil, water, and vegetation. Although it seldom causes disease in healthy people, P. aeruginosa is an opportunistic pathogen associated with fatal pneumonia in patients with CF, as well as patients with compromised immune systems and chronic infections such as non-cystic fibrosis bronchiectasis and urinary tract infections.

In CF patients, the initially colonizing P. aeruginosa strains are nonmucoid but in the CF lung, after a variable period, often one or two years, they inevitably convert into the mucoid form. Mucoid strains of P. aeruginosa grow as biofilms in the airways of CF patients (Yu, H., and N. E. Head, Front Biosci. 7:D442-57 (2002)). Biofilms refer to surface-attached bacterial communities encased in a glycocalyx matrix (Costerton, J. W., et al., Science 284:1318-22 (1999)).

Mucoi’d P. aeruginosa biofilms are microcolonies embedded in a capsule composed of copious amounts of alginate, an exopolysaccharide (Govan, J. R., and V. Deretic, Microbiol. Rev. 60:539-74 (1996)) and are resistant to host defenses (Ramsey, D. M., and D. J. Wozniak, Mol. Microbiol. 56:309-22 (2005)).

The emergence of mucoid strains of P. aeruginosa in CF lungs signals the beginning of the chronic phase of infection and is associated with further disease deterioration and poor prognosis (Lyckz, J. B., et al., Clin. Microbiol. Rev. 15:194-222 (2002)). The chronic phase of infection due to P. aeruginosa is characterized by pulmonary exacerbations (fever, elevated white blood cell count, increased sputum production, and decreased pulmonary function) that require antimicrobial therapy (Miller, M. B., and Gilligan, P. H., J. Clin. Microbiol. 41:4009-4015 (2003)). CF exacerbations are typically interspersed with intervening periods of relative quiescence, with each phase lasting various lengths of time (Miller, M. B., and Gilligan, P. H., J. Clin. Microbiol. 41:4009-4015 (2003)). However, lung function continuously declines, the infecting strains become increasingly resistant, and inevitably, the patient succumbs to cardiopulmonary failure (Miller, M. B., and Gilligan, P. H., J. Clin. Microbiol. 41:4009-4015 (2003)).

There is a growing consensus that the lung pathology that occurs during chronic P. aeruginosa infection is due to a large extent to the immune response directed against pseudomonal biofilms (Miller, M. B., and Gilligan, P. H., J. Clin. Microbiol. 41:4009-4015 (2003)). High levels of cytokines and leukocyte-derived proteases can be detected in airway fluid from CF patients and are believed to be responsible for much of the lung damage that occurs in this patient population (Miller, M. B., and Gilligan, P. H., J. Clin. Microbiol. 41:4009-4015 (2003)). Alginate appears to protect P. aeruginosa from the consequences of this inflammatory response as it scavenges free radicals released by activated macrophages (Simpson, J. A., et al., Free Rad. Biol. Med. 6:347-353 (1989)). The alginate mucoid coating also leads to the inability of patients to clear the infection, even under aggressive antibiotic therapies, most probably because it provides a physical and chemical barrier to the bacterium (Govan and Deretic, Microbiol. Rev. 60:539-574 (1996)).

Early aggressive antibiotic treatment of the initial colonizing non-mucoid P. aeruginosa population might prevent or at least delay chronic pulmonary infection. However, questions still remain as to whether such treatment should be performed routinely or only during pulmonary exacerbation, and whether the regimen could potentially lead to the emergence of resistant strains (Ramsey and Wozniak, Mol. Microbiol. 56:309-322 (2005)). Since P. aeruginosa is inherently resistant to many antibiotics at concentrations that can be achieved in vivo, with the exception of ciprofloxacin, those to which it is sensitive need to be given intravenously (Wilson and Dowling, Thorax 53:213-219 (1998)). However, long-term, aggressive antibiotic treatment is not without side effects. Therefore, it would be more beneficial to place the emphasis on aggressive treatment strategies before the in vivo switch to mucoidy since once chronic infection is established, it is rarely possible to eradicate it even with intensive, antibiotic therapy. Thus, early detection of conversion to mucoidy in patients is desired to allow aggressive therapy, thereby preventing further disease deterioration.

There is a significant and urgent need in hospitals and clinical laboratories for a rapid, sensitive and accurate diagnostic test for detection of potential conversion to mucoidy of *P. aeruginosa* prior to the detection of the emergence of a mucoid colony morphology on a growth plate in a laboratory.

**BRIEF SUMMARY OF THE INVENTION**

The present invention describes the identification and use of mucE, a positive regulator of alginate production in *P. aeruginosa*. Induction of mucE causes mucoid conversion in *P. aeruginosa*.

One object of this invention is to provide compositions for the early detection and diagnosis of the conversion to mucoidy of *Pseudomonas aeruginosa*. The present invention also provides molecular probes to detect the conversion from the nonmucoid to the mucoid state, via Northern blot, RT-PCR, or real-time RT-PCR, including diagnostic kits for the early detection of this disease state.

Another object of this invention is to provide methods for the early detection and diagnosis of the conversion to mucoidy of *Pseudomonas aeruginosa*. One method for detecting a cell converted to mucoidy involves obtaining a cell sample suspected of conversion to mucoidy, contacting messenger RNA from the cell sample with a mucE nucleic acid segment, and detecting the presence of increased hybridized complexes, wherein the presence of increased hybridized complexes is indicative of conversion to mucoidy. A six-fold increase of mucE messenger RNA is sufficient to cause conversion to mucoidy in mucA+ wild type cells. Thus, early detection of conversion to mucoidy is possible by detecting and measuring mucE expression as compared to the baseline expression level of mucE in non-mucoid cells.

Early detection for the trend of increased expression of the mucE message in various samples, including the sputum samples from patients with cystic fibrosis, samples from patients carrying endotracheal tubes, and urinary tract catheters would provide an indication that the colonizing bacteria has started to enter the biofilm mode of growth, thereby requiring immediate administration of aggressive antibiotic therapy.

A further embodiment of this invention is the use of MucE antibodies and methods of using MucE antibodies for detecting the conversion to mucoidy of *P. aeruginosa*.

A further embodiment of this invention is a method for preventing the conversion to mucoidy of *P. aeruginosa* by blocking mucE expression or mucE activity. Mucoid *P. aeruginosa* biofilms can be formed via two means: the mutations in mucA (see U.S. Pat. Nos. 6,426,187, 6,083,691, and 5,591,838), and increased expression of mucE. mucE acts upstream of mucA, thus, the control of mucoidy mediated by mucE occurs before the mucA mutation. Therefore, inhibition of MucE activity provides a means to prevent conversion to mucoidy during the early stage of bacterial colonization.

In still further embodiments, the present invention concerns a method for identifying new compounds that inhibit mucE gene expression or MucE function, which may be termed “candidate substances.” Such compounds may include anti-sense oligonucleotides or molecules that block or repress the mucE promoter, or molecules that directly bind to MucE to block the activity of MucE.

The present invention also provides a method for screening a candidate substance for preventing *P. aeruginosa* conversion to mucoidy comprising contacting *E. coli* bacteria with an effective amount of a candidate substance; and assay-
ing for reporter gene activity, wherein a decrease in the expression of the reporter gene indicates inhibition of mucE promoter activity.

Another object of the present invention is AlgW, a positive regulator for alginate production, and the use of AlgW as a potential drug target.

**BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1** shows the nucleotide sequence of mucE in *P. aeruginosa* (SEQ ID NO: 1). The mucE gene is an unclassified ORF (PA4633) in the genomes of PA01 and PA14. It encodes a small peptide of 89 amino acids with a molecular mass of 9.5 kDa.

**FIG. 2** shows the amino acid sequence of MucE in *P. aeruginosa* (SEQ ID NO: 2). MucE has a predicted N-terminal leader peptide of 36 amino acids, which is likely to direct the native peptide of MucE to the inner membrane for processing and export to the periplasm or outer membrane of *P. aeruginosa*. The WVF at the C-terminus is the signal for alginate induction.

**FIG. 3** shows the nucleotide sequence of the homolog of mucE in *P. aeruginosa* fluorescence Pr-5 (SEQ ID NO: 3). **FIG. 4** shows the amino acid sequence of the homolog of MucE in *P. aeruginosa* fluorescence Pr-5 (SEQ ID NO: 4). **FIG. 5** shows the number of mariner transposition insertions per TA site in the algU promoter region of four strains of *P. aeruginosa*. **FIG. 5A** shows the frequency of the insertions in each *P. aeruginosa* strain. **FIG. 5B** shows the sequence of the algU promoter region (SEQ ID NO: 16) containing all TA sites with an assigned number matching to **FIG. 5A**.

**FIG. 6** shows the levels of alginate, AlgU and MucB in *P. aeruginosa mucO* mutants caused by induction of algumucABC in comparison with the wild type PA01 (B). **FIG. 6A** shows the amounts of alginate (µg alginate/mg protein) that were measured for 4-72 h. Asterisk indicates significant differences at P<0.05 in comparison with the same time point in PA01. **FIG. 6B** is a Western blot analysis of the total protein extracts from the same cells as above by probing with anti-AlgU (Schorr, M. J., et al., *J. Bacteriol.* 178:4997-5004 (1996)) and anti-MucB (Boucher, J. C., et al., *J. Bacteriol.* 178:511-23 (1996)) monoclonal antibodies. The genotype of each mutant is shown. The number below each blot was the ratio of internally normalized AlgL and MucB to those of PA01 at the same time point. The +E superscript used in **FIG. 6A** refers to the overexpression of algU mucABC operon.

**FIG. 7** shows the levels of alginate, the expression of AlgU and MucB in V2E (PA01 mucE") as detected by Western blots (**FIG. 7A**) and RT-PCR (**FIG. 7B**). Bacterial cells were grown under the same conditions as described in Methods, and were subjected to the same treatments as in **FIG. 6**. Asterisk in alginate production indicates significant differences compared with PA01 at the same time point as in **FIG. 6**. The ratio of internally normalized AlgU and MucB to those of PA01 is shown.—in **FIG. 7B** indicates the RT minus controls.

**FIG. 8** shows upregulation of AlgU in VE13 (PA01 KinB") (SEQ ID NO: 29) in association with increased alginate production. **FIG. 8A** shows the levels of AlgU and MucB in various mutants after 24 h growth. FRD2 carries the algT18 suppressor mutant while FRD2-VE1 is like VE1 with the insertion in the algU promoter. VE3-NM1 to -NM4 are the spontaneous nonmucoid mutants with suppressors inactivating algU. VE3NM5+algU: pUCP20-algU in trans. VE22: cupS" and VE24: oprL" but with reduced expression of oprL due to production of the antisense RNA.

**FIG. 9** shows the regulatory cascade of alginate production in *P. aeruginosa*. AlgU is the alginate-specific sigma factor, whose activity is antagonized by anti-sigma factor, MucA. MucA is an inner membrane protein with its C-terminus in the periplasm, and its N-terminus interacting with AlgU in cytoplasm. The alginate operon consists of 12 genes encoding biosynthetic enzymes, thus collectively termed "alginate engine." The enzymes AlgI, AlgJ, and AlgK are involved in O-acetylation of alginate. AlgK is needed for formation of the alginate polymer and AlgE for the export of alginate across the membrane.

**FIG. 10** is a map of the expression vector pUCP20-Gmu-mucE. The expression vector contains the coding region of the mucE gene driven by a promoter derived from the gentamicin (Gm) cassette of pFAC. This promoter is highly expressive in *P. aeruginosa*. This construct can render the nonmucoid PA01 mucoid while the control backbone vector without mucE has no effect on the phenotype.

**FIG. 11** shows an alignment of the mucE homologs identified from the completed and partially completed genomes of three species within the genus of *Pseudomonas*. The three species are PA: *Pseudomonas aeruginosa*; PF: *Pseudomonas fluorescens*; and PS: *Pseudomonas syringae*. The strains shown are: PA-PA01 (SEQ ID NO: 23), *Pseudomonas aeruginosa* PA01 (causes opportunistic infections in humans); PA-PA14 (SEQ ID NO: 22), *Pseudomonas aeruginosa* UCBPP PA14 (human clinical isolate); PA-2192 (SEQ ID NO: 20), *Pseudomonas aeruginosa* 2192 (CF patient isolate); PA-C3719 (SEQ ID NO: 21), *Pseudomonas aeruginosa* C3719 (unknown source but probably clinical origin); PS-PPH (SEQ ID NO: 26), *Pseudomonas syringae* pv. phaseolicola 1448A (causes halo blight on beans); PS-PTO (SEQ ID NO: 27), *Pseudomonas syringae* pv. tomato DC3000 (bacterial speck disease on tomato plants); PS-SB728 (SEQ ID NO: 28), *Pseudomonas syringae* pv. syringae B728a (brown spot disease on beans); PF-PF5 (SEQ ID NO: 24), *Pseudomonas fluorescens* Pf-5 (Saprophyte) (the production of a number of antibiotics as well as the production of siderophores by this strain can inhibit phytopathogen growth); and PF-PFO1 (SEQ ID NO: 25), *Pseudomonas fluorescens* PfO-1 (micro-organism of putrefaction and well adapted to soil environments).

**FIG. 12** shows an alignment of the algal homologs identified from the completed and partially completed genomes of three species within the genus of *Pseudomonas*. The three species are PA: *Pseudomonas aeruginosa*; PF: *Pseudomonas fluorescens*; and PS: *Pseudomonas syringae*. All these species have the capacity to overproduce alginate. The strains shown are the same as for **FIG. 11**, and include AlgW homologs for PA-2192 (SEQ ID NO: 29), PA-C3719 (SEQ ID NO: 30), PA-PA14 (SEQ ID NO: 31), PA-PA01 (SEQ ID NO: 32), PF-PF5 (SEQ ID NO: 33), PF-PFO1 (SEQ ID NO: 34), PS-PPH (SEQ ID NO: 35), PS-PTO (SEQ ID NO: 36), and PS-SB728 (SEQ ID NO: 37). The predicted functional domains of AlgW include an N-terminal signal peptide sequence at amino acids 1-27, a trypsin domain (peptidase activity, serine at AlgW 227 is conserved) at amino acids 114-260, and a PDZ domain at amino acids 270-380.

**FIG. 13** shows the detection of N-terminal His-tag labeled MucE protein via Western Blot with anti-penta-his monoclonal antibody and SDS-PAGE with Coomassie blue.
FIG. 14 shows the sequence of mucE (SEQ ID NO: 2; amino acid sequence of mucE) and the phenotypes of the different translational mucE-phoA fusions (SEQ ID NO: 17; nucleic acid sequence of the full-length mucE-phoA fusion). The location of the mariner transposon bearing the aacC1 gene conferring Gm' in the chromosome of the mucoid mutants PAODVE2 and PA14DR4 is shown. Different lengths of mucE sequences were fused with phoA without the leader signal peptide sequence to demonstrate the effect of the signal sequence on translocation across the inner membrane to the periplasm. 1. Negative control, no 5' leader peptide sequence (no sig phoA); 2. Positive control, the wild-type E. coli phoA leader sequence restored in the construct by directly fusing it with phoA (Ec wt-phoA); 3. Full-length mucE-phoA; 4. mucE with the predicted N-terminal leader sequence fused with phoA; 5. partial mucE N-terminal leader sequence fused with phoA; 6. C-terminal mucE with ATG fused with phoA. The exact phoA fusion sites are as indicated in the mucE sequence. The leader sequence of mucE with max cleavage site is between pos. 36 (P) and 37 (A) (box).

FIG. 15 shows an alignment of MucP (SEQ ID NO: 19) and the Escherichia coli orthologue RseP (SEQ ID NO: 18). Identical amino acids are marked by an asterisk (*). The two terminal protease domains are shown in light gray and the two PDZ domains are shown in medium gray. The overlapping region containing both a portion of the protease domain and a portion of the PDZ domain is shown in dark gray.

DETAILED DESCRIPTION OF THE INVENTION

Infections due to P. aeruginosa are recognized by the medical community as particularly difficult to treat. In particular, the emergence of a mucoid phenotype of P. aeruginosa in CF lungs is associated with further disease deterioration and poor prognosis. A patient’s prognosis for recovery from an infection caused by mucoid P. aeruginosa is enhanced when the diagnosis is made and appropriate treatment initiated as early in the course of infection as possible before the number of bacteria in the host becomes overwhelming and much more difficult to bring under control. Thus, early detection of P. aeruginosa conversion to mucoidy in patients is particularly desired to allow aggressive therapy, thereby preventing further disease deterioration.

The present application describes the identification of a positive regulator involved in alginate and biofilm production in P. aeruginosa, termed mucE (SEQ ID NOs:1-2) (GenBank accession numbers DQ352561 (PAO1 mucE) and DQ352562 (PA14 mucE)). Induction of mucE causes mucoid conversion in P. aeruginosa.

One object of this invention is to provide compositions for the early detection and diagnosis of the conversion to mucoidy of Pseudomonas aeruginosa in biological specimens. By “early detection” is meant detecting P. aeruginosa conversion to mucoidy using certain assay methods, including but not limited to, methods involving the use of a nucleic acid probe or antibodies, 1 to 14 days, specifically 1 to 10 days, more specifically 1 to 7 days, and most specifically 6 days, 5 days, 4 days, 3 days, 2 days, 24 hours, 18 hours, 12 hours or 8 hours before detecting the emergence of a mucoid colony morphology on a growth plate in a laboratory.

The present invention also provides molecular probes to detect the conversion from the nonmucoid to the mucoid state, including via Northern blot, RT-PCR, or real-time RT-PCR, including diagnostic kits for the early detection of this disease state.

The present invention is also directed to algW and the use of AlgW as a potential drug target. Contrary to previous findings, AlgW is a positive regulator for alginate production. The algW gene and AlgW protein, the algW homologs, and the uses thereof as described above for the P. aeruginosa mucE gene and MucE protein are also part of the present invention.

Another object of this invention is mucA mucoid mutants and the use of these mutants to screen for suppressors and potential toxin genes. Mucoid mutants with mucA mutations (see U.S. Pat. Nos. 6,426,187, 6,083,691, and 5,591,838) have been previously detected from clinical specimens. The presence of these mutations is a poor prognosticator and represents the onset of chronic infection. Since the elevation of mucE can cause the emergence of mucoid P. aeruginosa before mucA mutations occur, the involvement of mucE in alginate induction is upstream of mucA.

Another object of this invention is to provide methods for the early detection and diagnosis of the conversion to mucoidy of Pseudomonas aeruginosa. One method for detecting a cell converted to mucoidy involves obtaining a biological specimen suspected of conversion to mucoidy, contacting messenger RNA from the specimen with a mucE nucleic acid segment, and detecting the presence of increased hybridized complexes, wherein the presence of increased hybridized complexes over baseline is indicative of conversion to mucoidy.

The biological specimen to be assayed for the presence of mucoid P. aeruginosa can be prepared in a variety of ways, depending on the source of the specimen. The specimen may be obtained from the following: patients with debilitated immune systems, sputum samples from patients with pneumonia, endotracheal samples from intubation patients under intensive care, samples from urinary catheters, samples from wounds, and especially from patients suffering from cystic fibrosis. Specimens may be a sample of human blood, sputum, wound exudate, endotracheal samples, respiratory secretions, human tissues (e.g., lung) or a laboratory culture thereof, and urine. Since alginate induction is synonymous with biofilm formation in vivo, the increased expression of mucE may also be used to monitor the biofilm formation in a confined environment during space travel (astronauts).

A further embodiment of this invention is the use of MucE antibodies and methods of using MucE antibodies for detecting the conversion to mucoidy of P. aeruginosa via ELISA or other immunoassays.

A further embodiment of this invention is a method for preventing the conversion to mucoidy of P. aeruginosa. In particular, the present invention concerns methods for identifying new compounds that inhibit mucE gene expression or MucE function, which may be termed “candidate substances.” Such compounds may include anti-sense oligonucleotides or molecules that block or repress the mucE promoter.

Specifically, when the last three amino acids of MucE are changed from WVF to other combinations, the majority of altered signals are ineffective to induce mucoid biofilm production, indicating the specificity of this signal in mucoid conversion. Thus, WVF is an important signal for mucoid biofilm formation in P. aeruginosa. This WVF signal plays a role in the bacterium’s ability to overproduce alginate and enter a biofilm mode of growth via regulated proteolysis as depicted in FIG. 9. The present invention provides for methods to employ the signal as a drug target. Diagnostic kits to screen for the presence of the signal in patients with chronic P. aeruginosa infections are contemplated. In addition, methods to screen for compounds that inhibit the function of this signal are also contemplated. Such compounds will have a specific anti-biofilm function.
The present invention also provides for a method for screening a candidate substance for preventing *P. aeruginosa* conversion to mucoidy comprising contacting *E. coli* bacteria with an effective amount of a candidate substance; and assaying for reporter gene activity, wherein a decrease in the expression of the reporter gene indicates inhibition of mucE promoter activity.

MucE homologs from other *Pseudomonas* species or strains are also contemplated (see FIG. 11). These *Pseudomonas* species and strains include PA-PAOI, *Pseudomonas aeruginosa* PA01 (causes opportunistic infections in humans); PA-PA14, *Pseudomonas aeruginosa* UCBPP PA14 (human clinical isolate); PA-2192, *Pseudomonas aeruginosa* 2192 (CF patient isolate); PA-C3719, *Pseudomonas aeruginosa* C3719 (unknown source but probably clinical origin); PS-PPH, *Pseudomonas syringae* pv. *phaseolicola* 1448A (causes halo blight on beans); PS-PS728, *Pseudomonas syringae* pv. *syringae* B728a (brown spot disease on tomato plants); PS-PPF5, *Pseudomonas fluorescens* Pf-5 (Saprophyte) (the production of a number of antibiotics as well as the production of siderophores by this strain can inhibit phytopathogen growth); and PF-PPF01, *Pseudomonas fluorescens* PFO1-1 (microorganism of putrefaction and well adapted to soil environments). The mucE homologs and the use thereof as described above for the *P. aeruginosa* mucE gene and MucE protein are also part of the present invention.

Isolated nucleic acids comprising fragments containing one or more mucE consensus regions are also contemplated. The consensus regions are shown in FIG. 11. By “isolated” nucleic acid is intended a nucleic acid molecule, DNA or RNA, circular or linear, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution.

The term “positive regulator” as used herein, means that the induction of expression and/or activity of such a gene encoding a functional protein causes alginate overproduction. Examples of positive regulators include algU, mucE, and algA.

The term “negative regulator” as used herein, means that the absence of such a gene encoding a functional protein causes alginate overproduction. Examples of negative regulators include kniB, mucA, mucB, and mucD.

The term “recombinant” as used herein, means that a protein is derived from recombinant (e.g., microbial) expression systems. The term “microbial” refers to recombinant proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product, the term “recombinant microbial” defines a protein produced in a microbial expression system which is essentially free of native endogenous substances. Protein expressed in most bacterial cultures, e.g., *E. coli*, will be free of glucan.

The term “DNA sequence” refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct. Preferably, the DNA sequences are in a quantity or concentration enabling identification, manipulation, and recovery of the sequence and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such sequences are preferably provided in the form of an open reading frame uninterrupted by internal nontranslated sequences. Genomic DNA containing the relevant sequences could also be used. Sequences of nontranslated DNA may be present 5' or 3' from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

The term “nucleotide sequence” refers to a heteropolymer of deoxyribonucleotides. DNA sequences encoding the proteins of this invention can be assembled from fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit.

The term “recombinant expression vector” refers to a replicable DNA construct used either to amplify or to express DNA which encodes the recombinant proteins of the present invention and which includes a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structure or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. Structural elements intended for use in yeast expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may optionally be subsequently cleaved from the expressed recombinant protein to provide a final product.

As used herein, the term “expression vector” refers to a construct made up of genetic material (i.e., nucleic acids). Typically, an expression vector contains an origin of replication which is functional in bacterial host cells, e.g., *Escherichia coli*, and selectable markers for detecting bacterial host cells comprising the expression vector. Expression vectors of the present invention contain a promoter sequence and include genetic elements as described herein arranged such that an inserted coding sequence can be transcribed and translated in prokaryotes or eukaryotes. In certain embodiments described herein, an expression vector is a closed circular DNA molecule.

The term “expression” refers to the biological production of a product encoded by a coding sequence. In most cases, a DNA sequence, including the coding sequence, is transcribed to form a messenger-RNA (mRNA). The messenger-RNA is then translated to form a polypeptide product which has a relevant biological activity. Also, the process of expression may involve further processing steps to the RNA product of transcription, such as splicing to remove introns, and/or post-translational processing of a polypeptide product.

The term “recombinant microbial expression system” means a substantially homogeneous monoculture of suitable host microorganisms, for example, bacteria such as *E. coli* or yeast such as *S. cerevisiae*, which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit as a component of a resident plasmid. Generally, cells constituting the system are the progeny of a single ancestral transformant. Recombinant expression systems as defined herein will express heterologous protein upon induction of the regulatory elements linked to the DNA sequence or synthetic gene to be expressed.

One embodiment of the present invention is a method of detecting conversion to mucoidy in *Pseudomonas aeruginosa* in a biological specimen comprising detecting MucE expression. A preferred embodiment is a method of detecting conversion to mucoidy in *Pseudomonas aeruginosa* having an active mucE gene product comprising the detection of the mucE messenger RNA in a sample suspected of conversion to
mucoidy. In this case, the sequence encodes an active gene product and the sequence is detected by hybridization with a complementary oligonucleotide, to form hybridized complexes. The presence of increased hybridized complexes is indicative of conversion to mucoidy in Pseudomonas aeruginosa. The complementary oligonucleotides may be 5'-TCAAAAACCCAGGCAACTGTCAGC-3', (SEQ ID NO:5) 5'-AGTACCGAAGGACGGCTGCGGT-3', (SEQ ID NO:6) or 5'-TTGCTAATCGCCGAAACCCAF-3' (SEQ ID NO:7).

A further embodiment of the present invention is the use of MucE antibodies and methods of using MucE antibodies for detecting the conversion to mucoidy of P. aeruginosa or for inhibiting MucE function.

In still further embodiments, the present invention concerns a method for identifying new compounds that inhibit transcription from mucE promoter, which may be termed "candidate substances." Such compounds may include anti-sense oligonucleotides or molecules that encourage repression of the mucE promoter. The present invention provides for a method for screening a candidate substance for preventing P. aeruginosa conversion to mucoidy comprising: contacting E. coli bacteria with an effective amount of a candidate substance; and assaying for reporter gene activity, wherein a decrease in the expression of the reporter gene indicates inhibition of mucE promoter activity.

In additional embodiments, the present invention also concerns a method for detecting mucoid Pseudomonas aeruginosa bacteria in a biological sample. The method comprises reacting a sample suspected of containing P. aeruginosa with a detergent, EDTA, and a monoclonal antibody or fragment thereof capable of specifically binding to MucE expressed by P. aeruginosa, separating the sample from unbound monoclonal antibody; and detecting the presence or absence of immune complexes formed between the monoclonal antibody and MucE.

Polynucleotides

The DNA sequences disclosed herein will also find utility as probes or primers in nucleic acid hybridization embodiments. Nucleotide sequences of between about 10 nucleotides to about 20 or about 30 nucleotides, complementary to SEQ ID NOs:1-4, will find particular utility, with even longer sequences, e.g., 40, 50, 100, even up to full length, being more preferred for certain embodiments. The ability of such nucleic acid probes to specifically hybridize to mucE encoding sequences will enable them to be of use in a variety of embodiments. For example, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Nucleic acid molecules having stretches of 10, 15, 20, 30, 50, or even of 100 nucleotides or so, complementary to SEQ ID NOs:1 and 3, will have utility as hybridization probes. These probes will be useful in a variety of hybridization embodiments, such as Southern and Northern blotting in connection with analyzing the complex interaction of structural and regulatory genes in diverse microorganisms and in clinical isolates from patients, including CF patients. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the complementary region may be varied, such as between about 10 and about 100 nucleotides, according to the complementary sequences one wishes to detect.

The use of a hybridization probe of about 10 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the Polymerase Chain Reaction (PCR) technology of U.S. Pat. No. 4,603,102 (herein incorporated by reference) or by introducing selected sequences into recombinant vectors for recombinant production.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of homologous, or heterologous genes or cDNAs. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by 0.02 M-0.15 M NaCl at temperatures of 50° C. to 70° C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating functionally related genes.

In certain instances, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate specific mutant mucE-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as 0.15 M-0.9 M salt, at temperatures ranging from 20° C. to 55° C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic, biotinylated, and chemiluminescent labels, which are capable of giving a detectable signal. Fluorophores, luminescent compounds, radioisotopes, and particles can also be employed. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, calorimetric indicator substrates are known which can be employed to provide a means visible to
the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

Longer DNA segments will often find particular utility in the recombinant production of peptides or proteins. DNA segments which encode peptide antigens from about 15 to about 50 amino acids in length, or more preferably, from about 15 to about 30 amino acids in length are contemplated to be particularly useful. DNA segments encoding peptides will generally have a minimum coding length in the order of about 45 to about 150, or to about 90 nucleotides.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared in accordance with the present invention which are up to 10,000 base pairs in length, with segments of 5,000, 3,000, 2,000 or 1,000 base pairs being preferred and segments of about 500 base pairs in length being particularly preferred.

It will be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NOs:1 and 3. Therefore, DNA segments prepared in accordance with the present invention may also encode biologically functional equivalent proteins or peptides which have variant amino acid sequences. Such sequences may arise as a consequence of codon redundancy or equivalency which are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged.

Further embodiments of the invention include vectors comprising polynucleotides, which comprise a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences of the vectors comprising polynucleotides described above.

Other embodiments of the invention include polynucleotides, which comprise a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences of the polynucleotides described above.

As a practical matter, whether any particular vector or polynucleotide is at least 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence according to the present invention, can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

Codon Optimization

As used herein, the term “codon optimization” is defined as modifying a nucleic acid sequence for enhanced expression in the cells of the vertebrate of interest, e.g., human, by replacing at least one, more than one, or a significant number of codons of the native sequence with codons that are more frequently or most frequently used in the genes of that vertebrate. Various species exhibit particular bias for certain codons of a particular amino acid.

In one aspect, the present invention relates to polynucleotide expression constructs or vectors, and host cells comprising nucleic acid fragments of codon-optimized coding regions which encode therapeutic polypeptides, and fragments, variants, or derivatives thereof, and various methods of using the polynucleotide expression constructs, vectors, host cells to treat or prevent disease in a vertebrate.

As used herein the term “codon-optimized coding region” means a nucleic acid coding region that has been adapted for expression in the cells of a given vertebrate by replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of that vertebrate.

Deviations in the nucleotide sequence that comprise the codons encoding the amino acids of any polypeptide chain allow for variations in the sequence coding for the gene. Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino acids (the remaining three codons encode signals ending translation). Many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six, whereas tryptophan and methionine are coded by just one triplet. This degeneracy allows for DNA base composition to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA.

Consensus Sequences

The present invention is further directed to expression plasmids that contain chimeric genes which express therapeutic fusion proteins with specific consensus sequences, and fragments, derivatives and variants thereof. A “consensus sequence” is, e.g., an idealized sequence that represents the amino acids most often present at each position of two or more sequences which have been compared to each other. A consensus sequence is a theoretical representative amino acid sequence in which each amino acid is the one which occurs most frequently at that site in the different sequences which occur in nature. The term also refers to an actual sequence which approximates the theoretical consensus. A consensus sequence can be derived from sequences which have, e.g., shared functional or structural purposes. It can be defined by
aligning as many known examples of a particular structural or functional domain as possible to maximize the homology. A sequence is generally accepted as a consensus when each particular amino acid is reasonably predominant at its position, and most of the sequences which form the basis of the comparison are related to the consensus by rather few substitutions, e.g., from 0 to about 100 substitutions. In general, the wild-type comparison sequences are at least about 50%, 75%, 80%, 90%, 95%, 96%, 97%, 98% or 99% identical to the consensus sequence. Accordingly, polypeptides of the invention are about 50%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the consensus sequence.

A "consensus amino acid" is an amino acid chosen to occupy a given position in the consensus protein. A system which is organized to select consensus amino acids can be a computer program, or a combination of one or more computer programs with "by hand" analysis and calculation. When a consensus amino acid is obtained for each position of the aligned amino acid sequences, then these consensus amino acids are "lined up" to obtain the amino acid sequence of the consensus protein.

As mentioned above, modification and changes may be made in the structure of the mucE coding region and still obtain a molecule having like or otherwise desirable characteristics. As used herein, the term "biological functional equivalent" refers to such proteins. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in the DNA sequence and nevertheless obtain a protein with like or even countervailing properties (e.g., antigenic v. agonistic). It is thus contemplated by the inventors that various changes may be made in the DNA sequence of mucE (or MucE proteins or peptides) without appreciable loss of their biological utility or activity.

Polypeptides

Further embodiments of the invention include polypeptides, which comprise amino acid sequences at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical, to any of the amino acid sequences of the polypeptides described above.

As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NOs:2 and 4 can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park 575 Science Drive, Madison, Wisconsin 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acids in the reference sequence are allowed.

Even though the invention has been described with a certain degree of particularity, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the foregoing disclosure. Accordingly, it is intended that all such alternatives, modifications, and variations which fall within the spirit and the scope of the invention be embraced by the defined claims.

The following examples are included for purposes of illustration only and are not intended to limit the scope of the present invention, which is defined by the appended claims. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow, represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Antibodies

Further embodiments of the invention include mucE and AlgW monoclonal antibodies and methods of using mucE and AlgW antibodies for the detection and diagnosis of mucoid P. aeruginosa in biological specimens. The methods comprise reacting a specimen suspected of containing mucoid P. aeruginosa with a MucE or AlgW monoclonal antibody or fragment thereof, separating the specimen from unbound antibody, and detecting the presence of immune complexes formed between the monoclonal antibody and the MucE or AlgW protein, as compared to non-mucoid control cells and therefrom determining the presence of mucoid P. aeruginosa. Novel hybrid cell lines are also provided which produce the monoclonal antibodies capable of specifically binding to the MucE or AlgW protein expressed in P. aeruginosa. When the monoclonal antibodies are labeled and combined with a solubilizing reagent, a specific and rapid direct test for mucoid P. aeruginosa is achieved.

The monoclonal antibodies of this invention can be prepared by immortalizing the expression of nucleic acid sequences which code for antibodies specific for MucE or AlgW of P. aeruginosa. This may be accomplished by introducing such sequences, typically cDNA encoding for the antibody, into a host capable of cultivation and culture. The immortalized cell line may be a mammalian cell line that has been transformed through onecogenesis, by transfection, mutation, or the like. Such cells include myeloma lines, lymphoma lines, or other cell lines capable of supporting the expression and secretion of the antibody in vitro. The antibody may be a naturally occurring immunoglobulin of a mammal other than human, produced by transformation of a lymphocyte, by means of a virus or by fusion of the lymphocyte with a neoplastic cell, e.g., a myeloma, to produce a hybrid cell line. Typically, the lymphoid cell will be obtained from a human immunized against MucE or a fragment thereof containing an epitopic site.

Monoclonal antibody technology was pioneered by the work of Kohler and Milstein, Nature 256:495 (1975). Monoclonal antibodies can now be produced in virtually unlimited quantities consistently and with a high degree of purity. These qualities facilitate the reproducibility and standardization of performance of diagnostic tests which are required in hospitals and other clinical settings.

Immunization protocols are well known and can vary considerably yet remain effective. See Goldberg, Monoclonal Antibodies: Principles and Practice, (1983) which is incorporated herein by reference Immunogenetic amounts of antigenic MucE preparations are injected, generally at concentrations in the range of 1 ug to 20 mg/kg of host. Administration of the antigenic preparations may be one or a plurality of times,
usually at one to four week intervals. Immunized animals are monitored for production of antibody to the desired antigens. The spleens are then removed and splenic B lymphocytes are isolated and transformed or fused with a myeloma cell line. The transformation or fusion can be carried out in conventional ways, the fusion technique being described in an extensive number of patents, e.g., U.S. Pat. Nos. 4,172,124; 4,350,683; 4,363,799; 4,381,292; and 4,423,147. See also Kennett et al., Monoclonal Antibodies (1980) and references therein.

The biological sample suspected of containing P. aeruginosa is combined with the primary antibody under conditions conducive to immune complex formation. If the test is a one-step immunofluorescence assay, the primary antibody will be labeled. Typically, the specimen is first fixed or adhered to a glass slide by heat and/or ethanol treatment, although other fixatives or adherents are known by those skilled in the art. The specimen is then contacted with the solubilizing agent for a sufficient period, usually from 1 to 30 minutes and more usually about 10 minutes, and the solubilizer is then washed from the slide. Alternatively, as described above, the solubilizing agent and the primary antibody may be combined and added as one step. The primary antibody should be incubated with the specimen for approximately 30 minutes at room temperature, although the conditions may be varied somewhat. The slide is rinsed to remove unbound antibody. If the primary antibody has been labeled with FITC, the reacted sample may be viewed under a fluorescence microscope equipped with standard fluorescein filters (excitation=490 nm; emission=520 nm) and a 40x oil immersion lens. The quantitation of fluorescence is based on visual observation of the brightness or relative contrast of the specifically stained antigen. Appropriate positive and negative controls make interpretation more accurate. A counterstain, such as Evans blue, may be employed to more easily visualize the fluorescent organisms.

The antibodies of the invention may be a chimeric antibody or fragment thereof, a humanized antibody or fragment thereof, a single chain antibody; or a Fab fragment.

Immunofluorescence staining methods can be divided into two categories, direct and indirect. In the direct staining method, a fluorochrome is conjugated to an antibody (the "primary antibody") which is capable of binding directly to the cellular antigen of interest. In the indirect staining mode, the primary antibody is not fluorescently labeled; rather, its binding is visualized by the binding of a fluorescently labeled second-step antibody, which second-step antibody is capable of binding to the primary antibody. Typically, the second-step antibody is an anti-immunoglobulin antibody. In some instances the second-step antibody is unlabeled and a third-step antibody which is capable of binding the second-step antibody is fluorescently labeled.

Indirect immunofluorescence is sometimes advantageous in that it can be more sensitive than direct immunofluorescence because for each molecule of the primary antibody which is bound, several molecules of the labeled second-step antibody can bind. However, it is well known that indirect immunofluorescence is more prone to nonspecific staining than direct immunofluorescence, that is, staining which is not due to the specific antigen-antibody interaction of interest (Johnson et al., in Handbook of Experimental Immunology, D. M. Weir, ed., Blackwell Publications Oxford (1979); and Selected Methods in Cellular Immunology, Mishell et al., ed., W. H. Freeman, San Francisco (1980)). In addition, the multiple steps involved in performing the indirect tests makes them slow, labor intensive, and more susceptible to technician error.

Various immunoassays known in the art can be used to detect binding of MucE or AlgW to antibodies, including but not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc.

Kits can also be supplied for use with the subject antibodies in the detection of mucoid P. aeruginosa in specimens, wherein the kits comprise compartments containing a MucE and/or AlgW monoclonal antibody capable of reacting with essentially all serotypes and immunotypes of P. aeruginosa, and labels and necessary reagents for providing a detectable signal. Thus, the monoclonal antibody composition of the present invention may be provided, usually in a lyophilized form, either alone or in conjunction with additional antibodies specific for other antigens of P. aeruginosa. The antibodies, which may be conjugated to a label, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., bovine serum albumin, or the like. Generally, these materials will be present in less than about 5% weight based on the amount of active antibody, and usually present in a total amount of at least about 0.001% weight based on the antibody concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1% to 99% weight of the total composition. Where a second antibody capable of binding to the monoclonal antibody is employed, this will usually be present in a separate vial. The second antibody may be conjugated to a label and formulated in a manner analogous to the antibody formulations described above.

Cystic Fibrosis (CF) Risk Assessment

Further embodiments of the invention include methods for Cystic Fibrosis (CF) disease assessment in an individual which comprise detecting the presence or absence of MucE and/or AlgW in a sample from an individual. Further embodiments include methods for Cystic Fibrosis (CF) disease assessment in an individual which comprise detecting the presence or absence of MucE or AlgW antibodies in a sample from an individual.

Additional embodiments include methods for treating P. aeruginosa biofilms in Cystic Fibrosis (CF) disease in an individual which comprise the steps of detecting the presence of MucE and/or AlgW in a sample from an individual; and selecting a therapy regimen for the individual based on the presence of MucE and/or AlgW. The P. aeruginosa biofilms in Cystic Fibrosis (CF) disease are treated by the therapy regimen. Also contemplated are methods for treating P. aeruginosa biofilms in Cystic Fibrosis (CF) disease in an
individual which comprise the steps of detecting the presence of MucE and/or AlgW antibodies in a sample from an individual; and selecting a therapy regimen for the individual based on the presence of MucE and/or AlgW antibodies. The P. aeruginosa biofilms in Cystic Fibrosis (CF) disease are treated by the therapy regimen.

As used herein, “individual” is intended to refer to a human, including but not limited to, children and adults. One skilled in the art will recognize the various biological samples available for detecting the presence or absence of MucE or AlgW in an individual, by any of which may be used herein. Samples include, but are not limited to, airway surface liquid, sputa, or combinations thereof; human blood, wound exudate, respiratory secretions, human tissues (e.g., lung) or a laboratory culture thereof, and urine. Moreover, one skilled in the art will recognize the various samples available for detecting the presence or absence of MucE or AlgW antibodies in an individual, any of which may be used herein. Samples include, but are not limited to, airway surface liquid, sputa, or combinations thereof; human blood, wound exudate, respiratory secretions, human tissues (e.g., lung) or a laboratory culture thereof, urine, and other body fluids, or combinations thereof.

As used herein, “assessment” is intended to refer to the prognosis, monitoring, delaying progression, delaying early death, staging, predicting progression, predicting response to therapy regimen, tailoring response to a therapy regimen, of Cystic Fibrosis disease based on the presence or absence of MucE, AlgW, MucE antibodies, or AlgW antibodies in a biological sample.

As used herein, “therapy regimen” is intended to refer to a procedure for delaying progression, delaying early death associated with Cystic Fibrosis disease and/or Pseudomonas aeruginosa in a Cystic Fibrosis individual. In one embodiment, the therapy regimen comprises administration of agonists and/or antagonists of MucE and/or AlgW. In another embodiment, the therapy regimen comprises agonists and/or antagonists of Pseudomonas aeruginosa.

One skilled in the art will appreciate the various known direct and/or indirect techniques for detecting the presence or absence of MucE or AlgW, any of which may be used herein. These techniques include, but are not limited to, amino acid sequencing, antibodies, Western blots, 2-dimensional gel electrophoresis, immunohistochemistry, autoradiography, or combinations thereof.

All references cited in the Examples are incorporated herein by reference in their entirety.

EXAMPLES

Materials and Methods

The following materials and methods apply generally to all the examples disclosed herein. Specific materials and methods are disclosed in each example, as necessary.


Bacterial Strains, Plasmids, Transposons and Growth Conditions

P. aeruginosa strains were grown at 37°C in Lennox broth (LB), on LB agar or Pseudomonas Isolation Agar (PIA, DIFCO) plates. When required, PIA plates were supplemented with carbenicillin, tetracycline, or gentamicin at a concentration of 300 ng/ml. E. coli strains were grown in LB broth, or LB agar supplemented with carbenicillin (100 µg/ml), tetracycline (15 µg/ml), gentamicin (13 µg/ml), or kanamycin (40 µg/ml), when required.

Transposon Mutagenesis

A standard Pseudomonas conjugation protocol was followed with the following modifications. E. coli SM10 λpir carrying pFAC and P. aeruginosa strains were grown in 2 ml LB broth overnight at 37°C and 42°C, respectively. The cell density of the cultures was measured by optical density at 600 nm and adjusted to a ratio of 1:1, which was equivalent to 8x10⁶ cells for matings. The mixed cultures were incubated on LB plates for 6 h at 37°C. The cells were harvested and washed in LB broth. The final cell mixtures in a volume of 1 ml were spread on 8 PIA plates (50 ml each) supplemented with gentamicin. The conjugal pairs were incubated at 37°C for 24 h for selection and screening exconjugants with a mucoid colony morphology. Such mutants were isolated and purified a minimum of 3 times. Mutants were frozen in 10% skim milk in a ~80°C freezer.

DNA Manipulations.

Two steps of polymerase chain reaction (PCR)-based cloning were used for general cloning purposes. First, the target genes were amplified by high-fidelity PCR using the appropriate primer sets containing the built-in restriction sites followed by cloning into pCR4-TOPO. The DNA fragments were digested by restriction enzymes, gel-purified, and transferred to the shuttle vector pUCP20. All recombinant plasmids were sequenced to verify the absence of mutations with M13 universal forward and reverse primers using an ABI 3130 Genetic Analyzer at the Marshall University School of Medicine Genomics Core Facility. PCR reactions were performed with MasterAmp™ Taq DNA Polymerase (Epicon)

In reverse PCR (iPCR)

The mariner transposon and its junction region in pFAC were sequenced. The sequence of the junction region including the inverted repeats in pFAC (SEQ ID NO:8) is as follows:
A multiple cloning site (MCS) was identified immediately outside the 3' end of the gentamicin cassette within the transposon. To map the insertion site, an iPCR protocol was developed to utilize this convenient MCS. A Pseudomonas genomic DNA was purified using a QIAamp genomic DNA kit. The DNA concentration was measured using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). Two µg DNA was digested by restriction enzymes Sall or PstI at 37° C. overnight followed by gel purification. The fragmented DNA was ligated to form the circularly closed DNA using the Fast-Link™ DNA ligation kit (Epicentre). A volume of 1 µl ligated DNA was used as template for PCR using GM50UT and GM30UT according to the condition as follows, 94° C. for 1 min, 34 cycles consisting of 94° C. for 1 min, 58° C. for 2 min, and 72° C. for 2 min, and a final extension step consisting of 72° C. for 8 min. After PCR, the products were analyzed on a 1% agarose gel. The PCR products were purified using the QIAquick PCR purification kits and sequenced using GM50UT as described above.

Alginic and Protein Assays

The alginic acid assay was based on a previously published method (Kautson, C. A., and A. Jeanes, Anal. Biochem. 24:470-481 (1968)) with the following modifications. P. aeruginosa and mutants were grown on 50 ml PIA plates in triplicate for a period of 72 h. At various time points, bacterial growth was removed from plates and re-suspended in 40 ml phosphate-buffered saline (PBS; pH 7.4). The optical density at 600 nm (OD_{600}) was recorded. The alginate standard curve was produced using D-mannuronic acid lactone (Sigma) in a range of 0-100 µg/ml. To measure the protein concentration, cell population of 10^9 to 10^10. Total RNA was isolated using the RiboPure™-Bacteria Kit (Ambion) followed by DNase I digestion and reverse transcription using the Superscript™ III Reverse Transcriptase kit (Invitrogen). The cDNA was amplified by PCR using specific primers and the IQ5™ Real-Time PCR system (Bio-Rad). The relative expression levels were calculated using the 2^{-ΔΔCt} method (Livak, K. et al., Science 288:551-554 (2000)).
Monoclonal Antibodies

The AlgU and MucB monoclonal antibodies used in the Examples are from previously published sources (Boucher et al., J. Bacteriol. 178:511-523 (1996); Schurr et al., J. Bacteriol. 178:4997-5004 (1996)) with a low level of cross-reactivity. The specificities of these antibodies are appropriate because the algU and mucB negative strains failed to display the respective AlgU and MucB proteins (FIG. 8). Furthermore, two non-specific proteins of 50 kDa and 75 kDa from MucB and AlgU blots respectively were used as convenient internal controls to normalize the protein levels.

Southern Hybridization

A 754 by PCR product was amplified from acc1 of pUCP30T using GM-F and GM-R primers, which was purified via gel extraction and labeled with digoxigenin as described by the manufacturer (Roche Molecular Biochemicals). Agarose gels were soaked in 0.25 N HCl for 30 min, rinsed in H2O, soaked in 1.5 M NaCl/0.5 M NaOH for 30 min and 1.5 M NaCl/0.5 M Tris-Cl, pH 8.0 for 30 min. A blotting apparatus (BIO-RAD Vacuum Blotter) was used with a filter paper wick, a Hybond-N+ membrane (Amersham Pharmacia Biotech), and transferred with 10xSSC transfer buffer for 2 h. After transfer, the membrane was rinsed in transfer buffer and UV cross-linked. Hybridization was done using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science) and labeled probe described above.

Western Blot Analysis

Forty µg of total protein was prepared by bead-beating 3x for 1 min with 5 min intervals on ice. The proteins were mixed with 2xSDS-PAGE sample buffer. A Precision Plus Protein Standard (Bio-Rad) was used as molecular mass ranging from 10 to 250 kD. Protein and standard were loaded into a Criterion pre-cast gel of linear gradient (10-15% Tris-HCl gel) (Bio-Rad) and was run in a Criterion Cell (Bio-Rad) at 60V for 4 h. The transfer onto a PVDF membrane was done in a Criterion Blotter (Bio-Rad) with CAPS buffer at 50V for 1 h. Primary antibodies were obtained using standard techniques. Horseradish Peroxidase-labeled secondary antibodies, goat anti-mouse IgG (H+L) and goat anti-rabbit IgG (H+L), were obtained from Pierce Biotechnologies and Kirkegaard & Perry Laboratories, respectively. Primary antibodies were diluted 1:1000 and secondary antibodies 1:5000 in TBS/Tween before application. ECL Western Blotting Detection System (Amersham Biosciences) was used to detect the protein of interest. X-ray film was exposed, and developed on an Alphatek AX390SE developer. The protein intensity was analyzed using a Chemidoc XRS system (Bio-Rad) and Quantity One software (Bio-Rad). These results were normalized against an internal protein within each sample. The relative expression level for each protein was then compared.
TABLE I

<table>
<thead>
<tr>
<th>Insertion</th>
<th>PA01</th>
<th>PA0579NM</th>
<th>PA14</th>
<th>FRD2</th>
<th>Stat</th>
<th>Freq</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA0762-algUpromoter</td>
<td>5 (23.8)</td>
<td>6 (60.0)</td>
<td>8 (72.7)</td>
<td>3 (100.0)</td>
<td>22</td>
<td>49</td>
</tr>
<tr>
<td>PA4033-mucE</td>
<td>1 (4.8)</td>
<td>1 (9.1)</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA4082-cupB5</td>
<td>1 (4.8)</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knockout+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA0762-algU</td>
<td>2 (9.5)</td>
<td>3 (30.0)</td>
<td>5</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA0973-oprL</td>
<td>1</td>
<td></td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA0763-mucA</td>
<td>1 (10.0)</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA0766-mucB</td>
<td>1 (4.8)</td>
<td>1 (9.1)</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA0766-mucD</td>
<td>9 (42.9)</td>
<td>1 (9.1)</td>
<td>10</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA0844-kinB</td>
<td>1 (4.8)</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># matings</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td># mutants screened</td>
<td>81,280</td>
<td>88,800</td>
<td>126,000</td>
<td>75,000</td>
<td>371,080</td>
<td></td>
</tr>
<tr>
<td># mucoid obtained</td>
<td>3218</td>
<td>31</td>
<td>4</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td># independent mutants</td>
<td>21</td>
<td>10</td>
<td>11</td>
<td>3</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>mutation freq</td>
<td>3.9 x 10^-4</td>
<td>2.0 x 10^-4</td>
<td>2.5 x 10^-4</td>
<td>5.3 x 10^-3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*+Induction configuration.*

The number of mutants obtained from each strain of *P. aeruginosa* is shown. Number inside a bracket denotes the percentage of a mutation in the total number of mutants within a strain.

Similar to another himar1 transposon vector of the same lineage but constructed for *M. tuberculosis* studies (Rubin, E. J., et al., *Proc Natl Acad Sci USA* 96:1645-1650 (1999)), the transposon end in pFAC has no termination sequences. Therefore, three types of mutations can be caused by the transposon in this vector depending on how and where it is inserted on the genome. As shown in Table 1, when inserted in the algU, mucE or cupB5 promoter region, the transposon used its own promoter (P_gw) (Wohlleben, W., et al., *Mol Gen Genet.* 217: 202-208 (1989)) to direct the expression of the downstream genes. Reduced (knockdown) expression occurred when the transposon was inserted in the intergenic region of algUmucA or immediately downstream of oprL with P_gw in the opposite direction with regard to the upstream algU or oprL. When the transposon was within the coding sequences, this produced stop codons away from insertion sites due to frameshift mutations, producing gene knockouts for mucA, mucB, mucD and kinB.

The mucoid phenotype poises a great demand for energy from the cells. The amount of alginate in mucoid mutants was initially lower than that of the wild-type strain PA01 (FIG. 6A-8A), suggesting that mucoid mutants may grow slower than the non-mucoid counterparts to compensate for the energy demand.

**Example 2**

The Majority of Insertions are within algUmucABCD and Result in Upregulation of AlgU

While all pFAC insertions were within five clusters (data not shown), the most frequent sites (49%) were in the algU promoters with the transposons situated in the induction configuration. Since the algUmucABC genes are co-transcribed (DeVries, C. A. & Ohman, D. E., *J Bacteriol* 176:6677-6687 (1994); Firoved, A. M. & Deretic, V., *J Bacteriol* 185:1071-1081 (2003)), the levels of AlgU and MucB were measured in these mutants. VE1, one of the representative promoter mutants as shown in FIG. 5, was grown on PIA plates for quantification of alginate and the protein levels of AlgU and MucB.

As the results show, compared to PA01, VE1 produced increased amounts of alginate from 24 to 72 h in concurrence with increased levels of AlgU and MucB (FIG. 6). The level of AlgU was higher than that of MucB (P < 0.005). AlgU and MucB reached the steady-state level at 4 h and remained so for the rest of the time points. The algU mutants in PA0579NM, PA14 and FRD2 were mucoid and displayed the same trend as VE1 regarding alginate production and protein levels of AlgU and MucB. These results indicate that the algU promoter mutations were gain-of-function and associated with an elevated level of AlgU.

Twenty eight percent of mucoid mutants had insertions in the coding regions of mucA, mucB and mucD (TABLE 1). The Alg+ phenotype of the mucD mutants (DR8, VE19, VE14 23 and VE12) was complemented to Alg+ by mucD or mucBCD in trans. VE3 and V1, the equivalent of a triple knockout of mucAB, in PA01 and PA0579NM respectively, were complemented to Alg by mucA, but not by mucBC or mucBCD, in trans. The Alg+ phenotype in mucE mutants of PA01 (VE8) and PA14 (DR1) was complemented to Alg+ by mucE, mucBC and mucBCD, in trans. These results suggest that the insertions in mucA, mucB and mucD are loss-of-function (null) mutations.

**Example 3**

mucE and cupB5 Encode Two Novel Positive Regulators of Alginate

Alginate is regulated by a signal transduction pathway. While ample information is available on the interaction between the sigma factor AlgU and trans-inner membrane anti-sigma factor MucA, it is unclear what and how periplasmic signals activate the AlgU pathway leading to alginate overproduction. MucE and CupB5 identified here are two candidates for such signals. VE2 and DR4 had two identical insertions 16 bps upstream of ATG of PA4033 in PA01 and PA14, respectively (data not shown). The transposon in both mutants was in the induction configuration (TABLE 1). PA4033 belongs to a class of unclassified open reading frames (ORF)
in the annotated genome of PAO1, and encodes a hypothetical peptide (89 aa) with a predicted molecular mass of 9.5 kDa. The protein has a leader sequence of 36 aa with the mature MucE protein exported to periplasm. In E. coli, the outer membrane pathway is activated via a similar signal transduction system in which an outer membrane porin, OmpC serves as an inducing signal. The carboxy-terminal signal of MucE (WVF) has a three consensus sequence as does OmpC (YQF) (Walsh, N. P., et al., Cell 113:61-71 (2003)) and CupB (NIW). The results show that alginate production in VE2 was increased after 24 h (FIG. 7A) in association with the increased levels of AlgU and MucB compared with PAO1 at all time points (FIG. 7A vs. FIG. 6B).
KinB is a negative regulator of alginate in PAO1

As a sensor-kinase, KinB is responsible for responding to some environmental signals and phosphorylating a response...
The oprL gene encodes a homolog of the peptidoglycan associated lipoprotein precursor (168 aa) with a predicted molecular mass of 18 kDa. OprL has a leader sequence of 24 aa which probably directs the mature protein to the outer membrane. Reduced expression of oprL in VE24 caused mucoid conversion in PA01, and was associated with a reduced level of AlgU and MucB (FIG. 8B).

Example 6
Nonmucoid Revertants in AlgU-Hyperactive Mutants were Caused by Suppressor Mutations Inactivating algU

Eleven percent of insertions were in the intergenic region between algU and mucA in the knockdown configuration (Table 1). The mutants of this category were hyper mucoid. The level of AlgU in VE3 was slightly reduced compared with that in PA01 (FIG. 8B). The abundance of algU mRNA in VE3 was 84% of that in PA01 based on RT-PCR (FIG. 7B). Four random spontaneous non-mucoid revertants of VE3, PA01-VE3-NM1-4, were isolated (GenBank accession numbers DQ352563, DQ352564, DQ352565, and DQ352566). Sequencing the algU gene in VE3-NM1, -NM2, -NM3 and -NM4 revealed that all carried a completely inactivated algU gene due to tandem duplications or a nonsense mutation. The nucleotide sequences of these four algU mutants are:

VE3-NM1 (SEQ ID NO: 10):

VE3-NM2 (SEQ ID NO: 11):

VE3-NM3 (SEQ ID NO: 12):

VE3-NM4 (SEQ ID NO: 13):

Reduced Expression of oprL Causes Mucoid Conversion in PA01

One mutant, VE24, had an insertion at the stop codon (TAA) of oprL (PA0973) in the knockdown configuration.
These mutations resulted in the disappearance of AlgU and MucB in these mutants (FIG. 8B). The suppressor mutants were complemented to Alg" by algU in trans. The complemented mutants, which restored the mucoid phenotype, caused the reappearance of AlgU (FIG. 8B). We also measured the AlgU-dependent PI promoter activity by fusing the AlgU promoter (AlgU promoter) to the lacZ gene on the chromosome (DeVries, C. A. & Ohman, D. E., J Bacteriol 176:6677-6687 (1994); Schurr, M. J., et al., J Bacteriol 176:3375-3382 (1995)).

Assay of the β-galactosidase activity indicated that the AlgU activity was 2348±156 units in NH1 (algU') and 16.0±5.5 units in NH2 (algU - ) while that of the promoterless control in NH2 was 146±34 units (P=1.2x10^-5) (data not shown).

PA0579 is a relatively unstable mucoid mutant of PA01 origin with an undefined muc-23 mutation. A spontaneous non-mucoid revertant, PA0579NM, was isolated which had an unknown suppressor mutation. The algUmucA alleles in PA0579 and PA0579NM were sequenced but no mutations were detected. To discern the pathway that regulated the mucoid phenotype in this strain, PA0579NM was mutagenized to screen for mucoid mutants. Three sites, the algU promoter, the algUmucA intergenic region and mucA, were targeted that reversed the phenotype to Alg" (Table 1). The highest frequency of mutations (60% within the strain) occurred in the algU promoters causing increased levels of AlgU and MucB in the same fashion as in VE1 in FIG. 8A (data not shown).

The results show that inactivation of mucA and mucB did not cause a marked induction in the amounts of AlgU and MucB to the same extent as the kinB, mucE and cupB5 mutants (FIG. 8B vs. 6A-8A). This supports the notion that the mucA and oprL genes negatively regulate the activity of AlgU (Fiored, A. M. & Deretic, V., J Bacteriol 185:1071-1081 (2003); Mathee, K., et al., J Bacteriol 179:3711-3720 (1997)).

Example 7

Upregulation of AlgU (AlgT) Causes Mucoid Conversion

The mucoid phenotype in clinical isolates of *P. aeruginosa* is unstable, and non-mucoid revertants arise spontaneously in the laboratory. Suppressor mutations in algT were the main cause of mucoid suppression in *P. aeruginosa* (DeVries, C. A. & Ohman, D. E., J Bacteriol 176:6677-6687 (1994); Schurr, M. J., et al., J Bacteriol 176:3375-3382 (1995)). FRD2 is a CF isolate which has a suppressor mutation in algT8 (DeVries, C. A. & Ohman, D. E., J Bacteriol 176:6677-6687 (1994)). FRD2 is a CF isolate which has a suppressor mutation in algT8 (DeVries, C. A. & Ohman, D. E., J Bacteriol 176:6677-6687 (1994)). Three rare mucoid mutants were identified in FRD2 (Table 1). They all had an insertion in front of algU, in the same manner as the algU promoter mutants in PA01 (VE1), PA14, and PA0579NM, which resulted in increased transcription of the algT18mucA22mucBC operon as confirmed by Western blots. (FIG. 84).

The rare FRD2 mucoid mutants coupled with the upregulation of AlgU support the notion that AlgU is the only sigma factor controlling the expression of algD in *P. aeruginosa* (FIG. 8). The results indicate that a suppressor nonmucoid mutant (FRD2) can revert back to a mucoid phenotype.
(FRD2-VE1) in *P. aeruginosa*. This observation may help to explain why the algU suppressors are prevalent in clinical isolates.

Analysis of the suppressor mutations in algU indicate that AlgU is required for alginate overproduction but is not an essential protein in *P. aeruginosa*.

**Example 8**

The Carboxyl Terminus of MucE Affects Mucoid Induction

The carboxyl-terminal signal of MucE (WVF) has a similar three consensus as sequence as OmpC (YQF) (Walsh et al., 2003). Searching for this motif in the known outer membrane protein database from PAOI did not identify any obvious *E. coli* OmpC homologs, indicating that mucE encodes a protein specific for induction of alginate. Other protein signals with such a function also exist. The C-terminal CupB5 carries the three amino acid motif NIW. NIW and WVF are not interchangeable in MucE (unpublished observation), indicating that MucE and CupB5 work on different effector proteins in the periplasm. Table II shows the effect of altering the carboxyl terminus of MucE on mucoid induction in *P. aeruginosa*.

**Table II**

<table>
<thead>
<tr>
<th>Carboxyl terminal sequences</th>
<th>Mucoid induction</th>
<th>Outer membrane proteins with the same C-terminal peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>WVF (Wild-type)</td>
<td>M</td>
<td>MucE</td>
</tr>
<tr>
<td>YVF</td>
<td>M</td>
<td>OprP, OprQ</td>
</tr>
<tr>
<td>LVF</td>
<td>M</td>
<td>MucE orthologue (<em>P. fluorescens</em>)</td>
</tr>
<tr>
<td>WIF</td>
<td>M</td>
<td>MucE orthologue (<em>P. syringae</em>)</td>
</tr>
<tr>
<td>WWV</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>WVF</td>
<td>NM</td>
<td></td>
</tr>
<tr>
<td>WQF</td>
<td>NM</td>
<td>OprS, HasR, OmpC and OmpF of <em>E. coli</em></td>
</tr>
<tr>
<td>WLF</td>
<td>NM</td>
<td></td>
</tr>
<tr>
<td>DRF</td>
<td>NM</td>
<td>AlgE</td>
</tr>
<tr>
<td>YVF</td>
<td>NM</td>
<td>Strongest signal in <em>E. coli</em></td>
</tr>
<tr>
<td>YKF</td>
<td>NM</td>
<td>OprH (PA178)</td>
</tr>
<tr>
<td>FQF</td>
<td>NM</td>
<td>AlgI</td>
</tr>
<tr>
<td>WWV</td>
<td>NM</td>
<td></td>
</tr>
<tr>
<td>WVF</td>
<td>NM</td>
<td></td>
</tr>
<tr>
<td>ELR (AWVF)</td>
<td>NM</td>
<td></td>
</tr>
<tr>
<td>RWV (AF)</td>
<td>NM</td>
<td></td>
</tr>
</tbody>
</table>

M: mucoid; NM: Non-mucoid; *Slightly mucoid after 1 day of incubation

The results in Table II show that the last three carboxyl-terminal amino acids of MucE, WVF, are critical for the ability of MucE to induce mucoid induction. Similarly, the WVF signal induced mucoidy in *P. fluorescens*. The WVF and YVF carboxyl terminal sequences significantly induced mucoidy, while the YQF carboxyl terminal sequence did not (data not shown). The envelope signal is well conserved among Pseudomonads. Therefore, *P. fluorescens* is an alternative producer when alginate will be used for human consumption.

**Example 9**

MucE Interacts with AlgW Resulting in Alginate Overproduction

AlgW (GenBank accession number (U29172) is a periplasmic serine protease in *P. aeruginosa*. Inactivation of algW on the chromosome of PAOI-VE2 causes this strain to become nonmucoid (Boucher, J. C., et al., *J. Bacteriol.* 178:511-523 (1996)). Reversion back to the mucoid state occurs when a functional copy of algW is brought into the cells. Similarly, the disruption of algW in PAO1 (PA01ΔalgW) prevents mucoid induction even when plasmid-borne mucE (pUCP20-Gm'-mucE) was in a state of overexpression. MucE is found to interact with AlgW causing alginate overproduction by increasing the expression and/or activity of AlgU.

Normally, AlgW is inactive because the functional domain (the trypsin domain) is covered with a PDZ domain of its own. Interaction between MucE and AlgW results in the release of the PDZ domain of AlgW. This interaction occurs via the carboxyl terminus of MucE, specifically the terminal amino acids WVF, resulting in the activation of AlgW. Activated AlgW degrades the carboxyl terminus of anti-sigma factor MucA. This action causes the release of AlgU into the cytoplasm, thereby activating alginate biosynthesis (see FIG. 9). AlgU is the sigma factor that drives alginate biosynthesis. Therefore, MucE is an inducing signal for alginate overproduction and the periplasmic target of MucE is AlgW (see Table III).

**Table III**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>Wild type</td>
<td>NM</td>
</tr>
<tr>
<td>VE2</td>
<td>PA01 over-expressing mucE</td>
<td>M</td>
</tr>
<tr>
<td>VE2 algW KO</td>
<td>VE2 algW knockout</td>
<td>NM</td>
</tr>
<tr>
<td>VE2 algW KO +</td>
<td>VE2 algW KO +</td>
<td>M</td>
</tr>
<tr>
<td>pUCP20algW</td>
<td>pUCP20 algW</td>
<td></td>
</tr>
</tbody>
</table>

The nucleotide sequence of algW (SEQ ID NO:14) is as follows:

```
ATGCCAAGAGCCCTGCTTTTCTCTGCCTGGCGCGCTGGCGCGAGG
AGCTGGCGCCGCTGGCAGCTGGAGTCGAGCCAGACCCACACAGTACATCT
GCATTCTGCTGACCAACACACGCTGGACCTGGCCGACCAAGATCATTG
CCGCCGCAGCTGGCGCTGGAGTACAGTACGTTAAGRCACACTGGCCG
GATGACCCCTGGCGCTGGGAGGTCCAGGCGATCGCTGGCGCTGGCG
GCCATCGACCGAGCTTGCCCTGGCGCTGGCAAGCGCTGGCCAGATGGCCATCGAT
CCGGCGGCTCCAGGCTATCGCTGCTGCAGCGCAACCTGGCGTTCAAGTCCAT
```

...
MucE is a small protein of about 9.5 kDa, predicted to be located in the periplasm. The results indicate that MucE functions upstream of the anti-sigma factor MucA. The interaction between DegS and OmpC, an outer membrane porin protein, has been shown to activate the signal transduction pathway for the activation of RpoE, the homolog of A1gU (Walsh, N. P., et al., Microbiology 151:2251-2261 (2005)). DegS is hypothesized to regulate the u stress response system by protease similar to HtrA in E. coli (PseudoCAP and Signal IP servers). The results suggest that MucE functions upstream of the anti-sigma factor MucA.

Example 10

The MucE Gene Encodes a Small Periplasmic or Outer Membrane Protein

The mucE gene is predicted to encode a polypeptide of 89 amino acids with a probable transmembrane helix and a cleavable N-terminal signal sequence. (Stover, C. K., et al., Nature 406:959-964 (2000)). Homologues of MucE are found in other species of pseudomonads capable of producing alginate (FIG. 11). We confirmed that mucE encodes a protein by detecting an approximately 10 kD protein in Western blots of cell extracts of E. coli and P. aeruginosa expressing His-tagged MucE (FIG. 13). PseudoCAP and Signal IP servers predicted that MucE is likely to be located in the periplasm. To test the localization of MucE, we constructed a series of deletions of mucE-phoA translational fusions. We observed phosphatase activity when phoA was fused to sequence corresponding to the full-length MucE or the N-terminus after P36 but not after A25. The MucE C-terminus-PhoA fusion did not show apparent phosphatase activity (FIG. 14). These results indicate that MucE is a small protein of about 9.5 kDa located in the periplasm or outer membrane, with an N-terminal signal sequence that is required for translocation across the cytoplasmic membrane. The homolog of AlgW is DegS in E. coli (see also FIG. 12). The interaction between DegS and OmpC, an outer membrane porin protein, has been shown to activate the signal transduction pathway for the activation of RpoE, the AlgU homolog in E. coli. It has been shown that interaction between OmpC and DegS in the periplasm activates the signal transduction pathway that controls the expression and/or activity of RpoE, a homolog of AlgU (Walsh, N. P., et al., Cell 113: 61-71 (2003)). The results suggest that MucE functions upstream of the anti-sigma factor MucA.

Example 11

MucP is Essential for MucE-Induced Conversion to Mucoidy

In E. coli, the degradation of RseA requires another protease called RseP (also known as YaeL) to cleave the anti-sigma factor RseA after it is cleaved by DegS (Alba, B. M., et al., Genes Dev 16:2156-2168 (2002); Kanehara, K., et al., Embo J. 22:6389-6398 (2003)). The P. aeruginosa genome also contains a homolog of RseP (PA3649, designated as MucP) (FIG. 15). The role of MucP in the degradation of MucA and activation of AlgU activity was examined. Inactivation of mucP in PAO1VE2 caused a loss of mucoidy. Furthermore, the plasmid pUCP20 (pUCP20-mucP) restored the mucoid phenotype in PAO1VE2mucP. Similarly, disruption of mucP in PAO1 prevented mucoid conversion when a high level of MucE was present from plasmid pUC20-Gmr-mucP. In addition, a higher level of MucA and a lower level of AlgU were present in PAO1VE2mucP as compared to PAO1VE2 (data not shown) was seen. These results indicate that MucP is required for MucE activation of AlgU activity.

Example 12

MucE-Induced Mucoidy does not Require the Prc Protease

The gene prc (PA3257) was recently identified as a regulator of alginate synthesis in P. aeruginosa and is predicted to encode a PDZ domain-containing periplasmic protease similar to a E. coli protease called Prc or Tsp (Reiling S. A., et al., Microbiology 151:2251-2261 (2005)). Prc appears to act to promote mucoidy in mucA mutants by degrading truncated forms of MucA found in mucoid mucA mutants (Reiling S. A., et al., Microbiology 151:2251-2261 (2005)). To test whether Prc plays a role in the activation of alginate production mediated by MucE, MucE was overexpressed in a strain lacking Prc and examined for mucoidy. Cells of the prc null mutant PAO1-184 (preC/tetR) carrying either MucE-overexpression plasmid pUCP20-Gmr-mucE or pUCP20-PGm-mucE were as mucoid as PAO1 cells carrying pUCP20-Gmr-mucE or pUCP20-PGm-mucE. These results suggest that Prc is not required for mucoidy induced by MucE and is consistent with Prc only acting against truncated forms of MucA.

Example 13

MucD Eliminates Signal Proteins that Activate AlgW and Other Proteases to Cleave MucA

The mucD gene (PA0766) is a member of the algU mucABCDD operon and is predicted to encode a serine protease similar to DegS (Boucher, C. J., et al., J. Bacteriol. 178:511-523 (1996)). MucD appears to be a negative regulator of mucoidy and AlgU activity (Boucher, C. J., et al., J. Bacteriol. 178:511-523 (1996)). The mariner transposon library screen confirmed this result because several mucoid mutants were isolated that had transposons inserted within the coding region of mucD. HtrA in E. coli has been hypothesized to regulate the O stress response system by removing misfolded proteins in the periplasm that can activate the DegS protease via the degradation of the anti-sigma factor RseA (Alba, B. M., et al., Genes Dev. 16:2156-2168 (2002); Kanehara, K., et al., Embo J. 22:6389-6398 (2003)). Therefore, it was determined whether MucD of P. aeruginosa acted in a similar manner as HtrA of E. coli. To test this,
overexpression of MucD in a strain overexpressing MucE was examined. Overexpression of mucD from the plasmid pUCP20-mucD partially suppressed the mucoid phenotype of the mucD-overexpressing strain PA01VE2. This result is consistent with the notion that MucD can aid in the elimination of misfolded OMPs including MucE. In addition, disruption of mucP in the mucoid mucD mutant PA01VE19 caused the loss of the mucoid phenotype. The mucoid phenotype of PA01VE19ΔmucP was restored when mucP was in trans. Loss of the mucoid phenotype from the mucD mutant PA01VE19 after the disruption of algW was not observed. The results suggest that MucD can act to remove misfolded proteins that activate proteases for degradation of MucA and that at least under certain conditions other proteases independent of AlgW can also initiate the cleavage of MucA.

**SEQUENCE LISTING**

```plaintext
<!-- Sequence Listing in plain text format -->
```

**US 8,399,649 B2**
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 4

1 4 Met Gly Asn Leu Leu Arg Lys Gly Gln Val Ala Leu Val Arg Ile Phe
5 10
20 25 Ser Gly Asp Arg Pro Val Arg Leu Ser Leu Met Leu Ala Ala Tyr
30
35 40 Leu Gly Ile Ser Ala Cys Thr Val Pro Ala Ser Thr Ala Gly Cys Cys
45
50 55 Gln Pro Ser Gly Ile Gly Gln Tyr Pro Ala Ser Ala Leu Pro Ala Gly
60
65 70 Ser Asp Ser Asn Leu Thr Leu Asp Ala Glu Pro Val Ile Gly Arg Thr
75 80
85 90 Ala Leu Pro Thr Asn Leu Gln Pro Pro Ala Pro Arg Trp Val Phe
95

<210> SEQ ID NO 5
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Synthetic complementary oligonucleotide sequence designed to form hybridized complexes

<400> SEQUENCE: 5

tcaaaacacc cagcgcaact cgtcacg

<210> SEQ ID NO 6
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Synthetic complementary oligonucleotide sequence designed to form hybridized complexes

<400> SEQUENCE: 6

agtagcgaag gacgggctgg cggt

<210> SEQ ID NO 7
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Synthetic complementary oligonucleotide sequence designed to form hybridized complexes

<400> SEQUENCE: 7

tttggctagc ccagcgggaac ccat

<210> SEQ ID NO 8
<211> LENGTH: 1473
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

<220> FEATURE:
<223> OTHER INFORMATION: Synthetic sequence of mariner transposon's junction region

<400> SEQUENCE: 8

acctacaccc cccggtattaa ttggctatgta cggggcgct cccatatgta ccctcgtgct gatcacc
gtcattctga gaatagtgta tgcggcgacc gagttgctct tgcccggcgt caatacggga
ataataccgcg ccacataaca ggttggctga taagtccccg gtctaacaaa gaaaaacaca
ntttttttttg aaatattgct ttatattat caticatgg cttcaagcc gtagctact cttaaaaaa
cgaattgacg cgtaatctct gcaattgaca taagcctgtt cggttcgtaa actgtaatgc 300
aagtagcgta tgcgctcacg caactggtcc agaaccttga ccgaacgcag cggtggtaac 360
ggcgcagtgg cggttttcat ggcttgtatat gactgttttt ttgtacagtc tatgcctcgg 420
gcatcacaag agcaagccgc ccgggcaaga gcaactcggt cgccgcatac 480
acttacttca gaatgacttg gttgagtact caccagtcac agaaaagcat cttacggatg 540
gcatgacagt aagagaatta tgcagtgctg ccataaccat gagtgataac actgcggcca 600
acttacttct gttggtccc ggcttgtatat gactgttttt ttgtacagtc tatgcctcgg 660
tcagcggac tcgattacc tgaggaacct gcctgctaat atcgagctag gctgtaatgc 720
tgcttcgca caaagccgcc tttggttcgc ttcgcggtct tggcgtgttc ctggcgtggt 780
gcgcgctaat atgagagcgt tgcgctcacg caactggtcc agaaccttga ccgaacgcag 840
ggcgcagtgg cggttttcat ggcttgtatat gactgttttt ttgtacagtc tatgcctcgg 900
gtggctcctt cgagtaaagc actgcggcca ccgggcaaga gcaactcggt cgccgcatac 960
cggccgcggc ccgcgtcctcg gtaccgggcc ccccctcgaagtctcgagta ctggcgtggt 1020
tgcttcgca caaagccgcc tttggttcgc ttcgcggtct tggcgtgttc ctggcgtggt 1080
gtggctcctt cgagtaaagc actgcggcca ccgggcaaga gcaactcggt cgccgcatac 1140
ggcgcagtgg cggttttcat ggcttgtatat gactgttttt ttgtacagtc tatgcctcgg 1200
gtggctcctt cgagtaaagc actgcggcca ccgggcaaga gcaactcggt cgccgcatac 1260
ggcgcagtgg cggttttcat ggcttgtatat gactgttttt ttgtacagtc tatgcctcgg 1320
ggcgcagtgg cggttttcat ggcttgtatat gactgttttt ttgtacagtc tatgcctcgg 1380
ggcgcagtgg cggttttcat ggcttgtatat gactgttttt ttgtacagtc tatgcctcgg 1440
cggccgcggc ccgcgtcctcg gtaccgggcc ccccctcgaagtctcgagta ctggcgtggt 1473
<210> SEQ ID NO 9
<211> LENGTH: 5622
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic sequence of pUCP20-Gm-MucE plasmid
<400> SEQUENCE: 9

gacgaaaggg cctcgtgata cgcctatttt tataggttaa tgtcatgata ataatggttt 60
ccttagtcgtc aggtggcact tttcggggaa atgtgcgcgg aacccctatt tgtttatttt 120
tctaaataca ttcaaatatg tatccgctca tgagacaata accctgataa atgcttcaat 180
aatattgaaa aaggaagagt atgagtattc aacatttccg tgtcgccctt attccctttt 240
ttgccgatt tttggttcgc ttttcttgct caccagaaac gctggtgaaa gtaaaagatg 300
ttgcttcgca caaagccgcc tttggttcgc ttcgcggtct tggcgtgttc ctggcgtggt 360
tctggctggc ttcgctggca cggagcaggt cttcgtcaac acgcgtgatg 420
tatggagcgt cggagcaggt cttcgtcaac acgcgtgatg 480
acactctca gtcatgacaggt cggagcaggt cttcgtcaac acgcgtgatg 540
gcagaacctg aagagacatta cgcggcggtc atttttcttg caccacagtt tgacgtgatg 600
actttttccc gacgtgatg ccccagttcc caccacagtt tgacgtgatg 660
gaggctgtc gcggcggtc atttttcttg caccacagtt tgacgtgatg 720
aacgtgagtc ccccagttcc caccacagtt tgacgtgatg 780
acactctca gtcatgacaggt cggagcaggt cttcgtcaac acgcgtgatg 840
ttgccaggac ccctcttcgct tgggccttcc cggctggctg gtttattgc

gagccggtga gcgtggctcgc gtcggtatca ttgcagcact ggggccagat ggtaagccct

cccctatcgc acgacgctgc ggacgacacc gcgtgctg

tgttctttcg ccacatgc aacaatcc ccataagctg gttttcgcc cactgagct

cagaccctcg agaaaaagatc aagacgagtg ttgacgagc gatcaagagc

ttcctctctcg ttccccgta gtaaccgcgc gcattgcctga tattctctg

gtagctgcgg gccgcacctgc ggggtgcttc ctccagcagaaa cagacgttgc

ggcagggag acgggagatc cgggcagttt catctcgagct ccgctgtgctg

cggccggagt gcctggctgc ggacgctcgc ctggtggtatc ccctctgctg

tgcctgctgc ccctctgctg ttcctgctgt ctggctgtcc cgggtttcgc

tgccgtgttc ttttttggca aaatcctgta tatcgtgcga aaaaagatgg atataccgaa

tgatttcctga gcttttattt attccacact gttcccttca agagcgatac ccctcgaatt

ggcgcggtcat gttttctttga gaagcgcctga ccagttgctgc gttttcgcc ctggttggc

tcgtctctgct aatcctgctt ccagtggctg ccgccagtgg cgataagtcg tgtcttaccg

ggtgctgctg ccctctgctg ttcctgctgt ctggctgtcc cgggtttcgc

tgccgtgttc ttttttggca aaatcctgta tatcgtgcga aaaaagatgg atataccgaa

tgatttcctga gcttttattt attccacact gttcccttca agagcgatac ccctcgaatt

ggcgcggtcat gttttctttga gaagcgcctga ccagttgctgc gttttcgcc ctggttggc

tcgtctctgct aatcctgctt ccagtggctg ccgccagtgg cgataagtcg tgtcttaccg

ggtgctgctg ccctctgctg ttcctgctgt ctggctgtcc cgggtttcgc

tgccgtgttc ttttttggca aaatcctgta tatcgtgcga aaaaagatgg atataccgaa

tgatttcctga gcttttattt attccacact gttcccttca agagcgatac ccctcgaatt

ggcgcggtcat gttttctttga gaagcgcctga ccagttgctgc gttttcgcc ctggttggc

tcgtctctgct aatcctgctt ccagtggctg ccgccagtgg cgataagtcg tgtcttaccg

ggtgctgctg ccctctgctg ttcctgctgt ctggctgtcc cgggtttcgc

tgccgtgttc ttttttggca aaatcctgta tatcgtgcga aaaaagatgg atataccgaa

tgatttcctga gcttttattt attccacact gttcccttca agagcgatac ccctcgaatt

ggcgcggtcat gttttctttga gaagcgcctga ccagttgctgc gttttcgcc ctggttggc

tcgtctctgct aatcctgctt ccagtggctg ccgccagtgg cgataagtcg tgtcttaccg
<210> SEQ ID NO 10
<211> LENGTH: 674
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic sequence of algU mutant, VE3-NM1

<400> SEQUENCE: 10

cgattcgctg ggacgctcga agctcctcca ggttcgaaga ggagctttca tgctaaccca 60

ggaacagac cagcaactgg tgcgaaggggt acagcgcgga gacaagcggg tttcgaatct 120
gtcgtaactg aataccagcc acagatact gcggatgtcg tgcgaagcg tgcgaacgc 180
ccaggaacgc cagcaactgg ccgaggaacc ttctcataag gcataccagtg cgtcgacgca 240
tttcgctggc gtagtgcctt tttataccttg gctgtatcgg atcgcagatca acacccgaa 300
gacaaaccttg gtcgctcgcc gcggctccagcc gacagagcag cagcacggg 360
gggcttcgcc ggacgctcga agctcctcca ggttcgaaga ggagctttca tgctaaccca 420

cccaagcctt ggtcataattgacggtcgc aggagagctcct gccctcgcgg 480
ttcgactgatc aatacctgg gtagttggcg gcgtggtcg ccgctgcagc 540
gagagctccttg ggtcataattgacggtcgc aggagagctcct gccctcgcgg 600
gagagctccttg ggtcataattgacggtcgc aggagagctcct gccctcgcgg 660
gacaaaccttg gtcgctcgcc gcggctccagcc gacagagcag cagcacggg 724
<210> SEQ ID NO 11
<211> LENGTH: 668
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic sequence of algU mutant, VE3-NM2

<400> SEQUENCE: 11

cctggcagac gtagcctcag gcgcgctcag gttgaacagag ggcctttcat 60

gtaaccccg gcaagaaccag cgcagctgtt tgcgaagggga cgagcgcgga gacaagcggg 120
ttcgactgatc aatacctgg gtagttggcg gcgtggtcg ccgctgcagc 180
gcgaagcgc cagcaactgg ccgaggaacc ttctcataag gcataccagtg cgtcgacgca 240
gacaaaccttg gtcgctcgcc gcggctccagcc gacagagcag cagcacggg 300
gagagctccttg ggtcataattgacggtcgc aggagagctcct gccctcgcgg 360
gagagctccttg ggtcataattgacggtcgc aggagagctcct gccctcgcgg 420
gagagctccttg ggtcataattgacggtcgc aggagagctcct gccctcgcgg 480
gacaaaccttg gtcgctcgcc gcggctccagcc gacagagcag cagcacggg 540
gagagctccttg ggtcataattgacggtcgc aggagagctcct gccctcgcgg 600
gagagctccttg ggtcataattgacggtcgc aggagagctcct gccctcgcgg 660
gacaaaccttg gtcgctcgcc gcggctccagcc gacagagcag cagcacggg 724
<210> SEQ ID NO 12
<211> LENGTH: 669
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic sequence of algU mutant, VE3-NM3

<400> SEQUENCE: 12

tattctgcca agaagcagtct ctgaggtgcc ccagctctcc agagagctct 60
tcatgctaac cccagaacag gatcagacaac tgttggaacq ggtacacagc ggagacaagc 120
gggctcttcga tctgtggtta ctaaataac ccagaacagat acctggagtt atctgtgcgc 180
tctgacgcaca gcgtccagggc ggtgctagcc tagcagcagct ggcacccgcg aagcagatcc 240
gtgcggtcgg ggtggtgctag atcgagtcggc atgttcagcgg ggacagccag gcagctgtga 300
tccacacgctc ctcgtggtcgc ggcgttctgc gggctttcggc gagcgaacat ggaatccgtc 360
cgagagggcg tctgctggta ctgaaatacc agcacaagat actgggattg atcgtgcggt 420
tcgtgacgctc gcgcctggctgc cgcctgctcc ggcgttccag gcgtctgtga ctcgacggtc 480
tgcgcagcctc tcagcctgcc ggcgttctgc cgcctgacgc gcgtctgtca ctcgacggtc 540
tgcgcagcctc tcagcctgcc ggcgttctgc cgcctgacgc gcgtctgtca ctcgacggtc 600
aagcaactgc caaagctcgg gcgcaaaatgc gcagcgaatgc gcagcgaatgc gcagcgaatgc 660
aagagagta 669

<210> SEQ ID NO 13
<211> LENGTH: 672
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE: OTHER INFORMATION: Synthetic sequence of algU mutant, VE3-NM4
<400> SEQUENCE: 13

gattcgctgg gacgctcgaa gctcctccag gttcgaagag gagctttcat gctaacccag 60
gacagggctc agcaagtgtg tgaacgggta cagcgcggag acaagcgggc tttcgatctg 120
tctggtactga aataccagca caagatactg ggattgatcg tgcggttcgt gcacgacgcc 180
caggaagccc aggacgtagc gcaggaagcc ttcatcaagg cataccgtgc gctcggcaat 240
tttccgcggcg atagtgcttt ttatacctgg ctgtatcgga tcgccatcaa caccgcgaag 300
aacacctcgg tctgctggcg ggcgtgcaac gcggacagct gcagcgaatgc gcagcgaatgc 360
gegctctcgg gcggcgacac gcgcggcgac gcggcgacac gcggcgacac gcggcgacac 420
cggtatgaga tgcgcgacgc ggccgtgctg ccgctgctg ccgctgctg ccgctgctg 480
cgacgacgctc cggcgtgggc ctgcggcggtg atcctgtgag cgctgctggt ccgctgctg 540
tgcgcagcctc tcagcctgcc ggcgttctgc cgcctgacgc gcgtctgtca ctcgacggtc 600
tgcgcagcctc tcagcctgcc ggcgttctgc cgcctgacgc gcgtctgtca ctcgacggtc 660
tgcgcagcctc tcagcctgcc ggcgttctgc cgcctgacgc gcgtctgtca ctcgacggtc 720

<210> SEQ ID NO 14
<211> LENGTH: 1170
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas aeruginosa
<400> SEQUENCE: 14

gattcgctgg gacgctcgaa gctcctccag gttcgaagag gagctttcat gctaacccag 60
tctggtactga aataccagca caagatactg ggattgatcg tgcggttcgt gcacgacgcc 120
tctggtactga aataccagca caagatactg ggattgatcg tgcggttcgt gcacgacgcc 180
tctggtactga aataccagca caagatactg ggattgatcg tgcggttcgt gcacgacgcc 240
tctggtactga aataccagca caagatactg ggattgatcg tgcggttcgt gcacgacgcc 300
tctggtactga aataccagca caagatactg ggattgatcg tgcggttcgt gcacgacgcc 360
tctggtactga aataccagca caagatactg ggattgatcg tgcggttcgt gcacgacgcc 420
tctggtactga aataccagca caagatactg ggattgatcg tgcggttcgt gcacgacgcc 480
cttaagaaco tgcgggcat gacccctcggc cgctccgacg gcattcgcac cggcgacgtc
540
tgcctcgcca tcggcaaccc gttcggcgtc ggccagaccg tgaccatggg catcatcagc
600
gccaccggac gcaaccagct cggcctgaac acctacgaag actcatacga gacccagcg
660
gcgatcaaco cggcagctc cgccggcgct gttggtgcgc tgtacctggtg gttcttctcg
720
atcaccaggg gtattttcttc caaagcgggtg cggcgcgtcgt cggcggcgcgt cggcgcgcgc
780
accaagctgg cctgagcttc cggcgttcgg caacagcgggt cggcggcgcgt cggcgcgcgc
840
tgtcggcggt ctcggttcgc cggcggcgcgt cggcggcgcgt cggcggcgcgt cggcgcgcgc
900
gacccgcgag cggcggcgcgt cggcggcgcgt cggcggcgcgt cggcggcgcgt cggcgcgcgc
960
tgctggccgg ctcgctgctc cttccagttt ccagttttcc ctgctcggcgg ctgctcggcgg
1020
togatgagcagctgcgttcgg cggcggcgcgt cggcggcgcgt cggcggcgcgt cggcgcgcgc
1080
aacgacagcagctgcgttcgg cggcgcgtcgc gcggcggcgcgt cggcgcgcgcgt cggcgcgcgc
1140
ccacagcagcagctgcgttcgg cggcgcgtcgc gcggcggcgcgt cggcgcgcgcgt cggcgcgcgc
1170

<210> SEQ ID NO 15
<211> LENGTH: 389
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas aeruginosa
<400> SEQUENCE: 15

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

Glu His Gly Gln Val Ile Arg Gly Trp Leu Gly Val Glu Val Lys Ala
275 280 285

Leu Thr Pro Glu Leu Ala Glu Ser Leu Gly Leu Gly Thr Ala Gly
290 295 300

Ile Val Val Ala Gly Val Tyr Arg Asp Gly Pro Ala Ala Arg Gly Gly
305 310 315 320

Leu Leu Pro Gly Asp Val Ile Leu Thr Ile Asp Lys Glu Glu Ala Ser
325 330 335

Asp Gly Arg Arg Ser Met Asn Gln Val Ala Arg Thr Arg Pro Gly Glu
340 345 350

Lys Ile Ser Ile Val Val Leu Arg Asn Gly Gln Lys Val Asn Leu Thr
355 360 365

Ala Glu Val Gly Leu Arg Pro Pro Pro Ala Pro Ala Pro Gln Gln Lys
370 375 380

Gln Asp Gly Gly Glu
385

<210> SEQ ID NO 16
<211> LENGTH: 547
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic algU promoter region
<400> SEQUENCE: 16
agtaggtcga gccctgcgac agttcgccct tgctgaggac ggcgatgcgc aggtgttccg
60
gaagggtcaa ggccagactc aggccggcgg cgccgctgcc gatgaccagt acatcgtgtt
120
gataatgttg gctcatgccc gcatttcccc gtggtggagc cctagtatat agaagggcct
180
ggcggtcgta ttagccaccc cgccgctgcc gcggtcccac gggcgccggcg ccggtccag
240
gcggccagtc tccggccggt tcgtgtgtgc cttcgttgtgc gcgcgcgcta
300
aagcaggatg ctcggcccg ccggccgccg ccggccgccc
ggcgtccagtc tggctgcctg ctcggccgct ctcggccgct ctcggccgct
360
tgctgcgtgg ccggccgccc ccggccgccc ccggccgccc
420
ggcgtccagtc tggctgcctg ctcggccgct ctcggccgct ctcggccgct
480
tgctgcgtgg ccggccgccc ccggccgccc ccggccgccc
540

<210> SEQ ID NO 17
<211> LENGTH: 306
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic mucE gene
<400> SEQUENCE: 17
agcgccagtc gcaccaacc ggttccggac ggttttcgac ggttttgttg
60
gtcgctcact tccggccgct ggttccggac ggttttgttg
120
ggctgttctg cttgctgctg cttgctgctg cttgctgctg
180
ggtgctgctg cttgctgctg cttgctgctg cttgctgctg
240
acccgacccgg cttgctgctg cttgctgctg cttgctgctg
300
<table>
<thead>
<tr>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met</td>
<td>Leu</td>
<td>Ser</td>
<td>Phe</td>
</tr>
<tr>
<td>16</td>
<td>20</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Val</td>
<td>Leu</td>
<td>Ile</td>
<td>Thr</td>
</tr>
<tr>
<td>35</td>
<td>40</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>Gly</td>
<td>Val</td>
<td>Arg</td>
</tr>
<tr>
<td>50</td>
<td>55</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>Arg</td>
<td>Arg</td>
<td>Thr</td>
</tr>
<tr>
<td>65</td>
<td>70</td>
<td>75</td>
<td>80</td>
</tr>
<tr>
<td>Pro</td>
<td>Leu</td>
<td>Gly</td>
<td>Tyr</td>
</tr>
<tr>
<td>85</td>
<td>90</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>Ala</td>
<td>Ala</td>
<td>Ala</td>
</tr>
<tr>
<td>100</td>
<td>105</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>Phe</td>
<td>Ala</td>
<td>Tyr</td>
</tr>
<tr>
<td>115</td>
<td>120</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Pro</td>
<td>Val</td>
<td>Val</td>
</tr>
<tr>
<td>130</td>
<td>135</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>Ala</td>
<td>Pro</td>
<td>Gly</td>
</tr>
<tr>
<td>145</td>
<td>150</td>
<td>155</td>
<td>160</td>
</tr>
<tr>
<td>Asp</td>
<td>Trp</td>
<td>Asp</td>
<td>Ala</td>
</tr>
<tr>
<td>165</td>
<td>170</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>Thr</td>
<td>Thr</td>
<td>Val</td>
</tr>
<tr>
<td>180</td>
<td>185</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Lys</td>
<td>Leu</td>
<td>Asp</td>
</tr>
<tr>
<td>195</td>
<td>200</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>Val</td>
<td>Ser</td>
<td>Ser</td>
</tr>
<tr>
<td>210</td>
<td>215</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>Leu</td>
<td>Glu</td>
<td>Val</td>
</tr>
<tr>
<td>225</td>
<td>230</td>
<td>235</td>
<td>240</td>
</tr>
<tr>
<td>Gln</td>
<td>Ala</td>
<td>Gly</td>
<td>Asp</td>
</tr>
<tr>
<td>245</td>
<td>250</td>
<td>255</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>Val</td>
<td>Thr</td>
<td>Phe</td>
</tr>
<tr>
<td>260</td>
<td>265</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>Leu</td>
<td>Glu</td>
<td>Val</td>
</tr>
<tr>
<td>275</td>
<td>280</td>
<td>285</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>Glu</td>
<td>Ser</td>
<td>Lys</td>
</tr>
<tr>
<td>290</td>
<td>295</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>Pro</td>
<td>Lys</td>
<td>Val</td>
</tr>
<tr>
<td>305</td>
<td>310</td>
<td>315</td>
<td>320</td>
</tr>
<tr>
<td>Tyr</td>
<td>Gly</td>
<td>Pro</td>
<td>Phe</td>
</tr>
<tr>
<td>325</td>
<td>330</td>
<td>335</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>Met</td>
<td>Lys</td>
<td>Leu</td>
</tr>
<tr>
<td>340</td>
<td>345</td>
<td>350</td>
<td></td>
</tr>
</tbody>
</table>
Val Lys Leu Asn Asn Leu Ser Gly Pro Ile Ser Ile Ala Lys Gly Ala
355 360
Gly Met Thr Ala Glu Leu Gly Val Val Tyr Tyr Leu Pro Phe Leu Ala
365 370 375 380
Leu Ile Ser Val Asn Leu Gly Ile Ile Asn Leu Phe Pro Leu Pro Val
380 385 390 395 400
Leu Asp Gly Gly His Leu Leu Phe Leu Ala Ile Glu Lys Ile Lys Gly
400 405 410 415
Gly Pro Val Ser Glu Arg Val Gin Asp Phe Cys Tyr Arg Ile Gly Ser
415 420 425 430
Ile Leu Leu Val Leu Leu Met Gly Leu Ala Leu Phe Asn Asp Phe Ser
430 435 440 445
Arg Leu
450

<210> SEQ ID NO 19
<211> LENGTH: 450
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic MucP peptide
<400> SEQUENCE: 19
Met Ser Ala Leu Tyr Met Ile Val Gly Thr Leu Val Ala Leu Gly Val
1 5 10 15
Leu Val Thr Phe His Glu Phe Gly His Phe Trp Val Ala Arg Arg Cys
20 25 30
Gly Val Lys Val Leu Arg Phe Val Gly Phe Gly Thr Pro Leu Val
35 40 45
Arg Trp His Asp Arg His Gly Thr Glu Phe Val Ala Ala Ile Pro
50 55 60
Leu Gly Tyr Val Lys Met Leu Asp Glu Ala Glu Ala Val Pro
65 70 75 80
Ala His Leu Leu Glu Gin Ser Phe Asn Arg Lys Thr Val Arg Gin Arg
85 90 95
Ile Ala Ile Val Ala Ala Gly Pro Ile Ala Asn Phe Leu Leu Ala Ile
100 105 110
Leu Phe Phe Trp Val Val Leu Leu Gly Ser Gin Gin Val Gin Val Arg Pro
115 120 125
Val Ile Gly Ser Val Ala Pro Glu Ser Leu Ala Ala Gin Ala Gly Leu
130 135 140
Glu Ala Gly Gin Glu Leu Leu Val Asp Gly Glu Pro Val Thr Gly
145 150 155 160
Trp Asn Gly Val Asn Leu Gin Leu Val Arg Arg Leu Gly Glu Ser Gly
165 170 175
Thr Leu Glu Val Arg Val Gin Ala Gly Ser Gin Val Asn Val Asp Ser Thr
180 185 190
His Gin Val Arg Leu Asp Gly Trp Leu Lys Gly Asp Asn Pro Asp
195 200 205
Pro Ile Ala Ser Leu Gly Ile Arg Pro Trp Arg Pro Ala Leu Pro Pro
210 215 220
Val Leu Ala Glu Leu Asp Pro Lys Gly Pro Ala Gin Ala Ala Gly Leu
225 230 235 240
Lys Leu Gly Asp Arg Leu Gin Ser Ile Asp Gly Ile Ala Val Asp Asp
245 250 255
Trp Gin Gin Val Val Asp Ser Val Arg Ala Arg Pro Gly Gin Arg Val
Gln Leu Lys Val Leu Arg Asp Gly Glu Val Leu Asp Val Ala Leu Glu
Leu Ala Val Arg Gly Glu Gly Lys Ala Arg Ser Gly Tyr Met Gly Ala
Gly Val Ala Gly Thr Glu Trp Pro Ala Glu Met Leu Arg Glu Val Ser
Tyr Gly Pro Leu Glu Ala Val Gly Gin Ala Leu Ser Arg Thr Trp Thr
Met Ser Leu Leu Thr Leu Asp Ser Ile Lys Lys Met Leu Leu Gly Glu
Leu Ser Val Lys Asn Leu Ser Gly Pro Ile Thr Ile Ala Lys Val Ala
Gly Ala Ser Ala Gin Ser Gly Val Gly Asp Phe Leu Asn Phe Leu Ala
Tyr Leu Ser Ile Ser Leu Gly Val Leu Asn Leu Pro Ile Pro Val
Leu Asp Gly Gly His Leu Leu Phe Tyr Leu Val Glu Trp Val Arg Gly
Arg Pro Leu Ser Glu Arg Val Gin Ala Trp Gly Met Gin Ile Gly Ile
Ser Leu Val Val Gly Val Met Leu Leu Ala Leu Val Asn Asp Leu Ser
Arg Leu

<210> SEQ ID NO 20
<211> LENGTH: 89
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas aeruginosa
<400> SEQUENCE: 20
Met Gly Phe Arg Pro Val Ser Gin Arg Leu Arg Asp Ile Asn Leu Gin
1  5 10  15
Ala Leu Gly Lys Phe Ser Cys Leu Ala Leu Val Leu Gly Leu Glu Ser
20  25 30
Val Ser His Pro Ala Gly Pro Val Gin Ala Pro Ser Phe Ser Gin Gly
35  40  45
Thr Ala Ser Pro Ser Phe Ala Thr Pro Leu Gly Leu Asp Gly Pro Ala
50  55  60
Arg Ala Arg Ala Glu Met Trp Asn Val Gly Leu Ser Gly Ala Val Ser
65  70  75  80
Val Arg Asp Glu Leu Arg Trp Val Phe
85

<210> SEQ ID NO 21
<211> LENGTH: 89
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas aeruginosa
<400> SEQUENCE: 21
Met Gly Phe Arg Pro Val Ser Gin Arg Leu Arg Asp Ile Asn Leu Gin
1  5 10  15
Ala Leu Gly Lys Phe Ser Cys Leu Ala Leu Val Leu Gly Leu Glu Ser
20  25 30
Val Ser His Pro Ala Gly Pro Val Gin Ala Pro Ser Phe Ser Gin Gly
35  40  45
Thr Ala Ser Pro Ser Phe Ala Thr Pro Leu Gly Leu Asp Gly Pro Ala
50  55  60
Arg Ala Arg Ala Glu Met Trp Asn Val Gly Leu Ser Gly Ala Val Ser
65  70  75  80
Val Arg Asp Glu Leu Arg Trp Val Phe
85
Thr Ala Ser Pro Ser Phe Ala Thr Pro Leu Gly Leu Asp Gly Pro Ala 50 55 60
Arg Ala Arg Ala Glu Met Trp Asn Val Gly Ser Gly Ala Val Ser 65 70 75 80
Val Arg Asp Glu Leu Arg Trp Val Phe 85

<210> SEQ ID NO 22
<211> LENGTH: 89
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas aeruginosa

<400> SEQUENCE: 22
Met Gly Phe Arg Pro Val Ser Gin Arg Leu Arg Asp Ile Asn Leu Gin 1 5 10 15
Ala Leu Gly Lys Phe Ser Cys Leu Ala Leu Val Leu Gly Leu Glu Ser 20 25 30
Val Ser His Pro Ala Gly Pro Val Gin Ala Pro Ser Phe Ser Gin Gly 35 40 45
Thr Ala Ser Pro Ser Phe Ala Thr Pro Leu Gly Leu Asp Gly Pro Ala 50 55 60
Arg Ala Arg Ala Glu Met Trp Asn Val Gly Leu Ser Gly Ala Val Ser 65 70 75 80
Val Arg Asp Glu Leu Arg Trp Val Phe 85

<210> SEQ ID NO 23
<211> LENGTH: 89
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas aeruginosa

<400> SEQUENCE: 23
Met Gly Phe Arg Pro Val Ser Gin Arg Leu Arg Asp Ile Asn Leu Gin 1 5 10 15
Ala Leu Gly Lys Phe Ser Cys Leu Ala Leu Val Leu Gly Leu Glu Ser 20 25 30
Val Ser His Pro Ala Gly Pro Val Gin Ala Pro Ser Phe Ser Gin Gly 35 40 45
Thr Ala Ser Pro Ser Phe Ala Thr Pro Leu Gly Leu Asp Gly Pro Ala 50 55 60
Arg Ala Arg Ala Glu Met Trp Asn Val Gly Leu Ser Gly Ala Val Ser 65 70 75 80
Val Arg Asp Glu Leu Arg Trp Val Phe 85

<210> SEQ ID NO 24
<211> LENGTH: 79
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 24
Met Asn Ser Ala Leu Leu Leu Leu Asn Ala Ile Ala Ile Ala Val Leu 1 5 10 15
Ala Ala Phe His Phe Gin Pro Ala Asp Asp Ala Pro Gly Gly Thr 20 25 30
Ser Phe Ala His Tyr Gin Gin Arg Leu Ala Pro Gin Leu Ala Val Met 35 40 45
Asn Thr Gin Ile Glu Pro Gly Ser Val Thr Arg Val Thr Gin Gly Lys
Ala Ser Gln Gln Pro Ala Ala Ala Ala Thr Glu Arg Trp Val Phe
Met Asn Ser Ala Leu Val Phe Ala Asn Ala Ile Ala Leu Ala Val Leu
Val Ala Phe His Phe His Asp Ser Gly Ala Ser Asp Thr Gin Val Thr
Asp Asp Ser Leu Gin Met Pro Arg Ala Glu Gin Arg Trp Val Phe
Ala Ser Val Gln Met Pro Arg Ala Glu Gin Arg Trp Val Phe

<210> SEQ ID NO 25
<211> LENGTH: 75
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 25
Met Asn Ser Ala Leu Val Phe Ala Asn Ala Ile Ala Leu Ala Val Leu
1 5 10 15
Met Gly Phe His Leu Val Pro Glu Asp Asn Glu Lys Val Ala Gly Arg
20 25 30
Met Pro His Tyr Leu Gln Val Gin Lys Ala Pro Gin Trp Ala Val Leu
35 40 45
Ser Asp Gln Ser Phe Ala Pro Gin Val Ser Ser Gin Ser Glu Gin Ala
50 55 60
Leu Pro Ala His Ser Thr Glu Arg Leu Val Phe
65 70 75

<210> SEQ ID NO 26
<211> LENGTH: 79
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas syringae

<400> SEQUENCE: 26
Met Asn Ser Ala Leu Val Phe Ala Asn Ala Ile Ala Leu Ala Val Leu
1 5 10 15
Val Ala Phe His Phe His Asp Ser Gly Ala Ser Asp Thr Gin Val Thr
20 25 30
Ser Asp Gin Ser Phe Ala Pro Gin Val Ser Ser Gin Ser Glu Gin Ala
50 55 60
Leu Pro Ala His Ser Thr Glu Arg Leu Val Phe
65 70 75

<210> SEQ ID NO 27
<211> LENGTH: 79
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas syringae

<400> SEQUENCE: 27
Met Asn Ser Ala Leu Val Phe Ala Asn Ala Ile Ala Leu Ala Val Leu
1 5 10 15
Val Ala Phe His Phe Gin Asp Ser Gly Ala Ser Asp Thr Gin Val Thr
20 25 30
Ser Asp Gin Ser Phe Ala Pro Gin Val Ser Ser Gin Ser Glu Gin Ala
50 55 60
Leu Pro Ala His Ser Thr Glu Arg Leu Val Phe
65 70 75

<210> SEQ ID NO 28
<211> LENGTH: 79
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas syringae
<400> SEQUENCE: 28

Met  Asn  Lys  Thr  Leu  Ser  Ala  Leu  Asn  Ala  Ala  Leu  Val  Ala  Leu
1      5     10    15

Val  Ala  Phe  His  Phe  Gln  Asp  Ser  Gly  Ile  Lys  Asp  Ala  Gin  Ala  Ile
20  25    30

Thr  Pro  Ala  Pro  Val  His  His  Gin  Ala  Pro  Lys  Leu  Ala
35  40    45

Ile  Met  Thr  Asp  Arg  Val  Ala  Ser  Ala  Ala  Met  Leu  Ala  Asn  Asp  Asp
50    55   60

Asp  Glu  Ser  Leu  Gin  Phe  Pro  Arg  Ala  Glu  Gin  Arg  Trp  Val  Phe
65  70    75

<210> SEQ ID NO 29
<211> LENGTH: 389
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas aeruginosa

<400> SEQUENCE: 29

Met  Pro  Lys  Ala  Leu  Arg  Phe  Leu  Gly  Trp  Pro  Val  Leu  Val  Gly  Val
1      5     10    15

Leu  Leu  Ala  Leu  Leu  Ile  Ile  Gin  His  Asn  Pro  Glu  Leu  Val  Gly  Leu
20    25    30

Pro  Arg  Gin  Glu  Val  His  Val  Glu  Gin  Ala  Pro  Leu  Leu  Ser  Arg  Leu
35    40    45

Gln  Glu  Gly  Pro  Val  Ser  Tyr  Ala  Asn  Ala  Val  Ser  Arg  Ala  Ala  Pro
50    55   60

Ala  Val  Ala  Asn  Leu  Tyr  Thr  Lys  Met  Val  Ser  Lys  Pro  Ser  His   65  70    75    80

Pro  Leu  Phe  Asp  Asp  Pro  Met  Phe  Arg  Phe  Phe  Gly  Asp  Aen  Leu
85    90    95

Pro  Gin  Gin  Lys  Arg  Met  Glu  Ser  Ser  Leu  Gly  Ser  Ala  Val  Ile  Met
100   105   110

Ser  Ala  Glu  Gly  Tyr  Leu  Leu  Thr  Asn  Asn  His  Thr  Ala  Gly  Ala
115   120   125

Asp  Gin  Ile  Ile  Val  Ala  Arg  Asp  Gly  Arg  Glu  Thr  Ile  Ala  Gin
130   135   140

Leu  Val  Gly  Ser  Asp  Pro  Glu  Thr  Asp  Leu  Ala  Val  Leu  Lys  Ile  Asp
145   150   155   160

Leu  Lys  Asn  Leu  Pro  Ala  Met  Thr  Leu  Gly  Arg  Ser  Asp  Gly  Ile  Arg
165   170   175

Thr  Gly  Asp  Val  Cys  Leu  Ala  Ala  Glu  Pro  Phe  Gly  Val  Gly  Gin
180   185   190

Thr  Val  Thr  Met  Gly  Ile  Ile  Ser  Ala  Thr  Gly  Arg  Asn  Gin  Leu  Gly
195   200   205

Leu  Aen  Thr  Tyr  Glu  Asp  Phe  Ile  Gin  Thr  Asp  Ala  Aen  Phe
210   215   220

Gly  Asn  Ser  Gly  Gly  Ala  Leu  Val  Asp  Ala  Ala  Asn  Ala  Ile  Gly
225   230   235   240

Ile  Aen  Thr  Ala  Ile  Phe  Ser  Lys  Ser  Gly  Ser  Gin  Gly  Ile  Gly
245   250   255

Phe  Ala  Ile  Pro  Thr  Lys  Leu  Ala  Leu  Glu  Val  Met  Gin  Ser  Ile  Ile
260   265   270

Glu  His  Gly  Gin  Val  Ile  Arg  Gly  Trp  Leu  Gly  Val  Glu  Val  Lys  Ala
275   280   285

Leu  Thr  Pro  Glu  Leu  Ala  Glu  Ser  Leu  Gly  Leu  Gly  Glu  Thr  Ala  Gly
<210> SEQ ID NO 30
<211> LENGTH: 389
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas aeruginosa

<400> SEQUENCE: 30
Met Pro Lys Ala Leu Arg Phe Leu Gly Trp Pro Val Leu Val Gly Val
1     5     10     15
Leu Leu Ala Leu Leu Ile Ile Gin His Asn Pro Glu Leu Val Gly Leu
20    25    30
Pro Arg Glu Val His Val Glu Glu Pro Leu Leu Ser Arg Leu
35    40    45
Gln Glu Gly Pro Val Ser Tyr Ala Asn Ala Val Ser Arg Ala Ala Pro
50    55    60
Ala Val Ala Ann Leu Tyr Thr Thr Lys Met Val Ser Lys Pro Ser His
65    70    75    80
Pro Leu Phe Asp Asp Pro Met Phe Arg Arg Phe Gly Glu Ser Leu
85    90   95
Pro Gln Glu Lys Arg Met Glu Ser Ser Leu Gly Ser Ala Val Ile Met
100   105   110
Ser Ala Glu Gly Tyr Leu Thr Asn Asn His Val Thr Ala Gly Ala
115   120   125
Asp Glu Ile Ile Val Ala Arg Asp Gly Arg Glu Thr Ile Ala Glu
130   135   140
Leu Val Gly Ser Asp Pro Glu Thr Asp Leu Val Leu Lys Ile Asp
145   150   155   160
Leu Lys Ann Leu Pro Ala Met Thr Leu Gly Arg Ser Asp Gly Ile Arg
165   170   175
Thr Gly Asp Val Cys Leu Ala Ile Gly Asn Pro Phe Gly Val Gly Glu
180   185   190
Thr Val Thr Met Gly Ile Ile Ser Ala Thr Gly Arg Asn Glu Val Leu Gly
195   200   205
Leu Asn Thr Tyr Glu Asp Phe Ile Glu Thr Asp Ala Ala Ile Asn Phe
210   215   220
Gly Asn Ser Gly Gly Ala Leu Val Asp Ala Gly Asn Leu Ile Gly
225   230   235   240
Ile Asn Thr Ala Ile Phe Ser Lys Ser Gly Gly Ser Gly Gly Ile Gly
245   250   255
Glu His Gly Glu Val Ile Arg Gly Trp Leu Gly Val Val Lys Ala
<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu Thr Pro Glu Leu Ala Glu Ser Leu Gly Leu Gly Glu Thr Ala Gly</td>
<td>275</td>
<td>280</td>
</tr>
<tr>
<td>Ile Val Val Ala Gly Val Tyr Arg Asp Gly Pro Ala Ala Arg Gly Gly</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Leu Leu Pro Gly Asp Val Ile Leu Thr Ile Asp Lys Gln Glu Ala Ser</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Asp Gly Arg Arg Ser Met Asn Gln Val Ala Arg Thr Arg Pro Gly Gln</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Lys Ile Ser Ile Val Val Ala Gly Val Tyr Arg Asp Gly Gly Gln Leu Thr</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>Ala Glu Val Gly Leu Arg Pro Pro Ala Pro Ala Pro Glu Gin Gin Lys</td>
<td>85</td>
<td>90</td>
</tr>
<tr>
<td>Gln Asp Gly Gly Glu</td>
<td>120</td>
<td>125</td>
</tr>
<tr>
<td>Seq ID NO 31</td>
<td>LENGTH: 389</td>
<td>TYPE: PRT</td>
</tr>
</tbody>
</table>