Lecture #5 - 21st Century Applications of Genetic Engineering

themes/concepts

1. Variety of Genetic Engineering Applications
2. Genetic Engineering in Bacteria
3. Release of Genetically-Engineered Bacteria into Environment - A case study
4. Genetic Engineering Yeast
5. Genetic Engineering “Pharm” Animals
6. Merging “Pharm” Genetic Engineering and Mammalian Cloning
7. Genetic Engineering other Animals & Regulatory Issues
8. Genetic Engineering Plants & GMO Controversy!
Applications of Genetic Engineering

Basic Sciences

1. Express protein and study protein structure and function in vivo; isolate and purify protein to study protein structure and function in vitro.

2. Use purified protein to make antibodies for medical purpose and/or make vaccines for the treatment of disease.

3. Find chromosomal location of cloned gene, determine gene copy number, and study gene structure.


5. Study gene structure, gene sequence, and gene expression in organs, tissues, and individual cells.

6. Create transgenic animals and gene knock-out animals to study gene function.

7. Scale-up production, isolation, and purification of therapeutic proteins (i.e., insulin, human growth hormone, and clot-dissolving proteins used to treat heart attacks) for use in humans as recombinant DNA products.

8. Use in human gene therapy.

9. Use in forensic applications such as DNA fingerprinting.


11. Create new, genetically engineered microorganisms, animals, and plants with a range of applications from waste-degrading microorganisms to disease-resistant plants and animals.

12. Gene used to alter bacteria for cleaning up toxic waste.


Figure 3.10 Applications of Recombinant DNA Technology
Figure 1.3 The Biotechnology Tree: Different Disciplines Contribute to Biotechnology. The basic sciences are the foundation or "roots" of all aspects of biotechnology. The central focus or "trunk" for most biotechnological applications is genetic engineering. Branches of the tree represent different organisms, technologies, and applications that "stem" from genetic engineering and bioinformatics, central aspects of most biotechnological approaches. Regulation of biotechnology occurs through governmental agencies like the FDA, USDA, EPA, and OSHA, whose roles and responsibilities will be defined in Chapter 12.
Fig. 14.1 The different ways that recombinant DNA technology has been exploited.
GENETIC ENGINEERING APPLICATIONS

Recombinant Nucleic Acids (DNA & RNA)
1. DNA Fingerprinting Probes/Templates - Forensics
2. DNA Probes/Templates - Genetic Disease Diagnosis, Paternity, Infectious Disease Pathogens
3. Gene Therapy
4. Anti-Sense/RNAi Drugs

Recombinant Viruses
1. Gene Therapy Vehicles
2. Vaccines

Recombinant Microbes (Bacteria & Fungi/Molds)
1. Biot factories / Metabolic Engineering - Synthesis of Industrial Molecules
2. Drug Production - Human Proteins & Antibiotics
3. Enzymes / Protein Engineering - Food & Industrial Applications
4. Waste Remediation

Recombinant Animals
1. Disease Models (Rat, Mouse)
2. Drug Production (Whole Animals & Cells)
3. Transgenic Fish for Food Production
4. Transgenic Mosquitoes / Malaria Control

Recombinant Plants
1. Improved Crops / Higher yields / Food production
2. Drug Production
3. Biofactories / Fuel
4. Bioremediation
5. Improved Foods / More Nutritious!
GENETIC ENGINEERING HAS "COME OF AGE"

[Diagram of different branches of genetic engineering]

Market data of some bioproducts (~2000, estimates)

<table>
<thead>
<tr>
<th>Product</th>
<th>Volume (t)</th>
<th>Value (€)</th>
<th>price/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>beer</td>
<td>130,000,000</td>
<td>330 billion</td>
<td>2.50 €/kg</td>
</tr>
<tr>
<td>ethanol</td>
<td>19,000,000</td>
<td>5 billion</td>
<td>0.25 €/kg</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>800,000</td>
<td>800 million</td>
<td>1.00 €/kg</td>
</tr>
<tr>
<td>citric acid</td>
<td>700,000</td>
<td>700 million</td>
<td>1.00 €/kg</td>
</tr>
<tr>
<td>detergent protease</td>
<td>100,000</td>
<td>300 million</td>
<td>3.00 €/kg</td>
</tr>
<tr>
<td>aspartame</td>
<td>10,000</td>
<td>50 million</td>
<td>5.00 €/kg</td>
</tr>
<tr>
<td>cephalosporins</td>
<td>5,000</td>
<td>2.5 billion</td>
<td>500.00 €/kg</td>
</tr>
<tr>
<td>tetracyclines</td>
<td>5,000</td>
<td>250 million</td>
<td>50.00 €/kg</td>
</tr>
<tr>
<td>insulin</td>
<td>8 t</td>
<td>1 billion</td>
<td>125.00 €/kg</td>
</tr>
<tr>
<td>erythropoietin</td>
<td>10 kg</td>
<td>4 billion</td>
<td>500 million €/kg</td>
</tr>
</tbody>
</table>

1.3 euro = 1 dollar
2/7/05

And is now A HUGE WORLD-WIDE BUSINESS
TABLE 6.2
Ten-Year Sales Forecast of the Value of DNA Technology Products in the United States.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Human therapeutics</td>
<td>7,555&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13,935 25,545</td>
<td>13</td>
</tr>
<tr>
<td>Human diagnostics</td>
<td>1,760</td>
<td>2,705 4,050</td>
<td>9</td>
</tr>
<tr>
<td>Agriculture</td>
<td>285</td>
<td>740 1,740</td>
<td>20</td>
</tr>
<tr>
<td>Nonmedical diagnostics</td>
<td>225</td>
<td>330 465</td>
<td>8</td>
</tr>
<tr>
<td>Totals</td>
<td>10,100</td>
<td>18,400 32,400</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Millions of 1996 dollars. Source: Consulting Resources Corp.

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$\sim 30 \text{ Billion}!$

1976 $\sim 0$

2004 $\sim 30 \text{ Billion}$

(NOT INCLUDING VALUATION OF BIOTECH COMPANIES)

$\sim 190 \text{ Billion}$

AN INDUSTRY IS BORN!
GENETIC ENGINEERING OF BACTERIA
Table 34.1 Bacteria

<table>
<thead>
<tr>
<th>Major Group</th>
<th>Typical Examples</th>
<th>Key Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ARCHAEABACTERIA</strong></td>
<td>Bacteria that are not members of the kingdom Eubacteria. Mostly anaerobic with unusual cell walls. Some produce methane. Others reduce sulfur.</td>
<td></td>
</tr>
<tr>
<td><strong>EUBACTERIA</strong></td>
<td>Gram-positive bacteria. Form branching filaments and produce spores, often mistaken for fungi. Produce many commonly used antibiotics, including streptomycin and tetracycline. One of the most common types of soil bacteria, also common in dental plaque.</td>
<td></td>
</tr>
<tr>
<td><strong>CHROMOGENOSES</strong></td>
<td>Sulfur bacteria, Nitrobacter, Nitrocoenos</td>
<td>Bacteria able to obtain their energy from inorganic chemicals. Most extract chemical energy from reduced gases such as H$_2$S (hydrogen sulfide), NH$_3$ (ammonia), and CH$_4$ (methane). Play a key role in the nitrogen cycle.</td>
</tr>
<tr>
<td><strong>CYANOBACTERIA</strong></td>
<td>Anaerobes, Nostoc</td>
<td>A form of photosynthetic bacteria common in both marine and freshwater environments. Deeply pigmented, often responsible for &quot;blooms&quot; in polluted waters.</td>
</tr>
<tr>
<td><strong>ENTEROBACTERIA</strong></td>
<td>Escherichia coli, Salmonella, Vibrio</td>
<td>Gram-negative, rod-shaped bacteria. Do not form spores; usually aerobic heterotrophs; cause many important diseases, including bubonic plague and cholera.</td>
</tr>
<tr>
<td><strong>GLOTHING AND BUDGING BACTERIA</strong></td>
<td>Mycobacterium, Chlamydomyces</td>
<td>Gram-negative bacteria. Exhibit gliding motility by secreting slimy polysaccharides over which masses of cells glide; some groups form upright multicellular structures carrying spores called fruiting bodies.</td>
</tr>
<tr>
<td><strong>PSEUDOMONADS</strong></td>
<td>Pseudomonas</td>
<td>Gram-negative heterotrophic rods with polar flagella. Very common form of soil bacteria; also contain many important plant pathogens.</td>
</tr>
<tr>
<td><strong>RICKETTSIAS AND CHLAMYDIA</strong></td>
<td>Rickettsia, Chlamydia</td>
<td>Small, gram-negative intracellular parasites. <em>Rickettsia</em> life cycle involves both mammals and arthropods such as flies and ticks; <em>Rickettsia</em> are responsible for many fatal human diseases, including typhus (<em>Rickettsia prowazekii</em>) and Rocky Mountain spotted fever. Chlamydial infections are one of the most common sexually transmitted diseases.</td>
</tr>
<tr>
<td><strong>TORCHENES</strong></td>
<td>Treponema</td>
<td>Long, coil-shaped cells. Common in aquatic environments; a parasitic form is responsible for the disease syphilis.</td>
</tr>
</tbody>
</table>
Expression vectors are used to make recombinant proteins in bacterial cells.

1. An expression vector includes the appropriate sequences for transcription and translation in the host cell.

2. A foreign gene is inserted at a restriction site.

3. E. coli is transfected with the expression vector.

4. The foreign gene is expressed in E. coli because the expression vector is present.

What switches? Terminators? Codon usage (for synthetic genes)?
1. Antibody binds to TPA as it is being made on polysomes, allowing TPA mRNA to be isolated.

2. Reverse transcription makes a cDNA copy (gene) for TPA.

3. TPA DNA is inserted into the expression vector.

4. E. coli is transfected with the vector.

5. TPA protein is made in large amounts.

6. TPA is injected into a stroke patient to dissolve the blood clot.

**Synthesizing tPA in Bacterial Cells**

17.14 Tissue Plasminogen Activator: From Protein to Gene to Pharmaceutical

TPA is a naturally occurring human protein that prevents blood from clotting. Its isolation and use as a pharmaceutical agent for treating patients suffering from blood clotting in the brain or heart—in other words, strokes and heart attacks—was made possible by recombinant DNA technology.
Figure 13-6 Expression of human insulin in E. coli. The two chains of insulin are made separately as fusion proteins with β-galactosidase. They are processed chemically and then mixed, and active insulin forms. (Copyright © 1992 by J. D. Watson, M. Gilmian, J. Witkowski, and M. Zoller, *Recombinant DNA*, 2d ed. Copyright © Scientific American Books.)

Figure 13-7 Expression of human growth hormone (hGH) in E. coli. (a) The human signal sequence is removed, enabling the protein to be produced in bacterial cells. The product contains an extra bacterial methionine. (b) A bacterial signal sequence that targets the protein for secretion to the outside can be added. In this method, the product has no extra methionine. (Copyright © 1992 by J. D. Watson, M. Gilmian, J. Witkowski, and M. Zoller, *Recombinant DNA*, 2d ed. Copyright © Scientific American Books.)

Many other Protein Classes - Enzymes for food processing, etc.
### Table 10.1 Some human proteins that have been produced by recombinant DNA technology for treating various disorders

<table>
<thead>
<tr>
<th>Protein</th>
<th>Disorder(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-Antitrypsin</td>
<td>Emphysema</td>
</tr>
<tr>
<td>Adrenocorticotropic hormone</td>
<td>Rheumatic diseases</td>
</tr>
<tr>
<td>B-cell growth factors</td>
<td>Immune disorders</td>
</tr>
<tr>
<td>Bactericidal/permeability-increasing protein</td>
<td>Infections</td>
</tr>
<tr>
<td>Brain-derived neurotrophic factor</td>
<td>Amyotrophic lateral sclerosis (Lou Gehrig's disease)</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Osteomalacia</td>
</tr>
<tr>
<td>Colony-stimulating factors</td>
<td>Cancer</td>
</tr>
<tr>
<td>Chorionic gonadotropin</td>
<td>Female infertility</td>
</tr>
<tr>
<td>Endorphins and enkephalins</td>
<td>Pain</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>Burns</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>Anemia, kidney disorders</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Hemophilia</td>
</tr>
<tr>
<td>Factor IX</td>
<td>Hemophilia</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>Growth defects</td>
</tr>
<tr>
<td>Growth hormone-releasing factor</td>
<td>Anemia</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>Diabetes</td>
</tr>
<tr>
<td>Insulin-like growth factor</td>
<td>Diabetes, renal failure</td>
</tr>
<tr>
<td>Interferons (α, β, γ)</td>
<td>Viral diseases, cancer, multiple sclerosis</td>
</tr>
<tr>
<td>Interleukins</td>
<td>Cancer, immune disorders</td>
</tr>
<tr>
<td>Interleukin-1 receptor</td>
<td>Asthma, rheumatoid arthritis</td>
</tr>
<tr>
<td>Lymphotoxin</td>
<td>Cancer</td>
</tr>
<tr>
<td>Macrophage-activating factor</td>
<td>Nerve damage</td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>Birthing</td>
</tr>
<tr>
<td>Relaxin</td>
<td>Insufficient plasma proteins</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>Growth defects</td>
</tr>
<tr>
<td>Somatotropin C</td>
<td>Thyroid cancer</td>
</tr>
<tr>
<td>Thyroid-stimulating hormone</td>
<td>Blood clots</td>
</tr>
<tr>
<td>Tissue plasminogen activator</td>
<td>Cancer</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>Ulcers</td>
</tr>
<tr>
<td>Urogastrone</td>
<td>Blood clots</td>
</tr>
<tr>
<td>Urokinase</td>
<td></td>
</tr>
</tbody>
</table>
Many recombinant proteins have been approved as drugs.

2002 list

<table>
<thead>
<tr>
<th>Compound</th>
<th>Company</th>
<th>Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII</td>
<td>Baxter Healthcare, Genetics Institute, Centeon, Bayer</td>
<td>Hemophilia A</td>
</tr>
<tr>
<td>Factor VIIa</td>
<td>Novo Nordisk</td>
<td>Some forms of hemophilia</td>
</tr>
<tr>
<td>Factor IX</td>
<td>Genetics Institute</td>
<td>Hemophilia B</td>
</tr>
<tr>
<td>Hirudin</td>
<td>Ciba Novartis, Europharm, Hoechst Marion Roussel</td>
<td>Venous thrombosis, heparin-associated thrombocytopenia</td>
</tr>
<tr>
<td>Tissue plasminogen activator</td>
<td>Genentech</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>Truncated tissue plasminogen activator</td>
<td>Galenus Mannheim, Boehringer Mannheim/Centocor</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>Insulin</td>
<td>Eli Lilly, Novo Nordisk, Hoechst AG</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Insulin analogues</td>
<td>Eli Lilly, Novo Nordisk, Aventis</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Human growth hormone</td>
<td>Eli Lilly, Genentech, Biotechnology General, Pharmacia, Upjohn, Novo Nordisk, Serono Laboratories</td>
<td>Growth hormone deficiency in children</td>
</tr>
<tr>
<td>Human growth hormone analogue</td>
<td>Genentech</td>
<td>Growth hormone deficiency in children</td>
</tr>
<tr>
<td>Human growth hormone</td>
<td>Serono Laboratories</td>
<td>AIDs-associated catabolism and wasting</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Novo Nordisk</td>
<td>Hypoglycemia</td>
</tr>
<tr>
<td>Thyrrotropin-α</td>
<td>Genzyme</td>
<td>Thyroid cancer</td>
</tr>
<tr>
<td>Follicle-stimulating hormone</td>
<td>Ares-Serono, Organon</td>
<td>Anovulation and superovulation</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>Amgen, Ortho Biotech, Boehringer-Mannheim</td>
<td>Anemia</td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>Ortho-McNeil Pharmaceuticals, Janssen-Cilag</td>
<td>Lower-extremity diabetic neuropathic ulcers</td>
</tr>
<tr>
<td>DNase I</td>
<td>Genentech</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>β-Glucocerebrosidase analogue</td>
<td>Genzyme</td>
<td>Gaucher disease</td>
</tr>
<tr>
<td>IFN-α₂</td>
<td>Hoffmann-La Roche, Schering-Plough</td>
<td>Hairy cell leukemia, hepatitis B and C</td>
</tr>
<tr>
<td>Synthetic type 1 IFN-α</td>
<td>Amgen, Yamanouchi Europe</td>
<td>Chronic hepatitis C</td>
</tr>
<tr>
<td>IFN-α₂₀</td>
<td>Schering-Plough</td>
<td>Hairy cell leukemia, genital warts, hepatitis B and C</td>
</tr>
<tr>
<td>IFN-β₁₆ analogues</td>
<td>Schering AG, Berlex Laboratories, Chiron</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>IFN-β₁₆</td>
<td>Biogen, Ares-Serono</td>
<td>Relapsing multiple sclerosis</td>
</tr>
<tr>
<td>IFN-γ₁₆</td>
<td>Genentech</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>IL-2 analogue</td>
<td>Chiron</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>IL-11 analogue</td>
<td>Genetics Institute</td>
<td>Prevention of chemotherapy-induced thrombocytopenia</td>
</tr>
</tbody>
</table>

Abbreviations: IFN, interferon; IL, interleuken.

**Must go through FDA clinical trials**

**Time x Expense!**
Figure 5.11 The Use of Antibiotics and Vaccines to Combat Infectious Diseases Caused by Microorganisms Even though the use of antibiotics and vaccines has decreased the incidence of human illness caused by microorganisms in the United States, new strains of microbes that show resistance to many popular antibiotics and vaccines are emerging. New antibiotics and vaccines are required to fight these microbes.

THE IMMUNE RESPONSE

1. Viruses infect the cell. Viral proteins are displayed on the cell surface.

2. Viruses and viral proteins on infected cells stimulate macrophages.


4. Interleukin-1 activates helper T cells, which release interleukin-2.

5. Interleukin-2 activates B cells and cytotoxic T cells.

6. Cytotoxic T cells bind to infected cells and kill them.

7. Activated B cells multiply.

8. Some B cells become memory cells.

9. Other B cells become antibody-producing factories.

10. Antibodies bind to viral proteins, some displayed on the surface of infected cells.

11. Macrophages destroy viruses and cells covered with antibodies.

FIGURE 37.20
Overview of the specific immune response.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Pathogen</th>
<th>Vector/Reservoir</th>
<th>Epidemiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthrax</td>
<td><em>Bacillus anthracis</em></td>
<td>Animals, including processed skins</td>
<td>Bacterial infection that can be transmitted through contact or ingested. Rare except in sporadic outbreaks; may be fatal.</td>
</tr>
<tr>
<td>Botulism</td>
<td><em>Clostridium botulinum</em></td>
<td>Improperly prepared food</td>
<td>Contracted through ingestion or contact with wound. Produces acute toxic poison; can be fatal.</td>
</tr>
<tr>
<td>Chlamydia</td>
<td><em>Chlamydia trachomatis</em></td>
<td>Humans, STD</td>
<td>Urogenital infections with possible spread to eyes and respiratory tract. Occurs worldwide; increasingly common over past 20 years.</td>
</tr>
<tr>
<td>Cholera</td>
<td><em>Vibrio cholerae</em></td>
<td>Human feces, plankton</td>
<td>Causes severe diarrhea that can lead to death by dehydration; 50% peak mortality if the disease goes untreated. A major killer in times of crowding and poor sanitation; over 100,000 died in KwaZulu in 1994 during a cholera outbreak.</td>
</tr>
<tr>
<td>Dental caries</td>
<td><em>Streptococcus</em></td>
<td>Humans</td>
<td>A dense collection of this bacteria on the surface of teeth leads to secretion of acids that destroy minerals in tooth enamel—sugar alone will not cause caries.</td>
</tr>
<tr>
<td>Diphtheria</td>
<td><em>Corynebacterium diphtheriae</em></td>
<td>Humans</td>
<td>Acute inflammation and lesions of mucous membranes. Spreads through contact with infected individual. Vaccine available.</td>
</tr>
<tr>
<td>Gonorrhoea</td>
<td><em>Neisseria gonorrhoeae</em></td>
<td>Humans only</td>
<td>STD, on the increase worldwide. Usually not fatal.</td>
</tr>
<tr>
<td>Hansen's disease (leprosy)</td>
<td><em>Mycobacterium leprae</em></td>
<td>Humans, feral armadillos</td>
<td>Chronic infection of the skin; worldwide incidence about 10-12 million, especially in Southeast Asia. Spread through contact with infected individuals.</td>
</tr>
<tr>
<td>Lyme disease</td>
<td><em>Borrelia burgdorferi</em></td>
<td>Ticks, deer, small rodents</td>
<td>Spread through bite of infected tick. Lesion followed by rash, fever, fatigue, pain, stiff neck, and headache.</td>
</tr>
<tr>
<td>Peptic ulcers</td>
<td><em>Helicobacter pylori</em></td>
<td>Humans</td>
<td>Originally thought to be caused by stress or diet, most peptic ulcers now appear to be caused by this bacterium; good news for ulcer sufferers as it can be treated with antibiotics.</td>
</tr>
<tr>
<td>Plague</td>
<td><em>Yersinia pestis</em></td>
<td>Fleas of wild rodents; rats and squirrels</td>
<td>Killed 3% of the population of Europe in the 14th century; endemic in wild rodent populations of the western U.S. today.</td>
</tr>
<tr>
<td>Pneumonia</td>
<td><em>Streptococcus</em>, <em>Mycoplasma, Chlamydia</em></td>
<td>Humans</td>
<td>Acute infection of the lungs, often fatal without treatment.</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Humans</td>
<td>An acute bacterial infection of the lungs, lymph, and membranes. Its incidence is on the rise, complicated by the development of new strains of the bacteria that are resistant to antibiotics.</td>
</tr>
<tr>
<td>Typhoid fever</td>
<td><em>Salmonella typhi</em></td>
<td>Humans</td>
<td>A systemic bacterial disease of worldwide incidence. Less than 300 cases a year are reported in the U.S. The disease is spread through contaminated water or foods (such as improperly washed fruits and vegetables). Vaccines are available for travelers.</td>
</tr>
<tr>
<td>Typhus</td>
<td><em>Rickettsia typhi</em></td>
<td>Lice, rats, humans</td>
<td>Historically a major killer in times of crowding and poor sanitation, transmitted from human to human through the bite of infected lice and fleas. Typhus has a peak untreated mortality rate of 50%.</td>
</tr>
</tbody>
</table>
## TABLE 5.5  POTENTIAL BIOLOGICAL WEAPONS

<table>
<thead>
<tr>
<th>Agent</th>
<th>Disease Threat and Common Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brucella</em> (bacteria)</td>
<td>Different strains of <em>Brucella</em> infect livestock such as cattle and goats. Can cause brucellosis in animals and humans. Prolonged fever and lethargy are common symptoms. Can be mild or life threatening.</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> (bacterium)</td>
<td>Anthrax. Skin form (cutaneous) produces skin surface lesions that are generally treatable. Inhalation anthrax initially produces flu-like symptoms leading to pulmonary pneumonia which is usually fatal.</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em> (bacterium)</td>
<td>Botulism. Caused by ingestion of food contaminated with <em>C. botulinum</em> or its toxins. Varying degrees of paralysis of the muscular system created by botulinum toxins are typical. Respiratory paralysis and cardiac arrest often cause death.</td>
</tr>
<tr>
<td>Ebola virus or Marburg virus</td>
<td>Both are highly virulent viruses that cause hemorrhagic fever. Symptoms include severe fever, muscle/joint pain, bleeding disorders.</td>
</tr>
<tr>
<td><em>Francisella tularensis</em> (bacterium)</td>
<td>Tularemia. Lung inflammation can cause respiratory failure, shock, and death.</td>
</tr>
<tr>
<td>Influenza viruses (a large highly contagious group)</td>
<td>Influenza (flu). Severity and outcome depend largely on the strain of the virus.</td>
</tr>
<tr>
<td><em>Rickettsiae</em> (several bacteria strains)</td>
<td>Different strains cause diseases such as Rocky Mountain Spotted Fever and Typhus.</td>
</tr>
<tr>
<td>Variola virus</td>
<td>Smallpox. Chills, high fever, backache, headache, and skin lesions.</td>
</tr>
<tr>
<td><em>Yersinia pestis</em> (bacterium)</td>
<td>“Black” Plague. High fever, headache, painful swelling of lymph nodes, shock, circulatory collapse, organ failure, and death within days after infection in a majority of cases.</td>
</tr>
<tr>
<td>Bacterium</td>
<td>Human Disease</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------------------------------------------------</td>
</tr>
<tr>
<td>Borelia burgdorferi</td>
<td>Lyme disease</td>
</tr>
<tr>
<td>Chlamydia trachomatis</td>
<td>Eye infections, genito-urinary tract infections (i.e., pelvic inflammatory disease)</td>
</tr>
<tr>
<td>Escherichia coli 0157:H7</td>
<td>Severe foodborne illness (diarrhea)</td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>Serious infections in children (eye, throat and ear infections, meningitis)</td>
</tr>
<tr>
<td>Helicobacter pylori</td>
<td>Stomach (gastric) ulcers</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>Listeriosis (serious foodborne illness)</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>Naisseria meningitidis (MC58)</td>
<td>Meningitis and blood infections</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Pneumonia, chronic lung infections</td>
</tr>
<tr>
<td>Rickettsia prowazekii</td>
<td>Typhus</td>
</tr>
<tr>
<td>Rickettsia conorii</td>
<td>Mediterranean spotted fever</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>Acute (short-term) respiratory infection</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>Plague</td>
</tr>
<tr>
<td>Vibrio cholerae</td>
<td>Cholera (diarrheal disease)</td>
</tr>
</tbody>
</table>


Bacillus anthracis - Anthrax
Staphylococcus aureus - Staph infections
Mycoplasma genitalium - STD
Shigella enterica - food poisoning
Salmonella typhimurium - food poisoning

...and more...
### Examples of Medically Important Viral Genomes That Have Been Sequenced Recently

<table>
<thead>
<tr>
<th>Virus</th>
<th>Human Disease or Illness</th>
<th>Year Sequenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ebola virus</td>
<td>Ebola hemorrhagic fever</td>
<td>1993</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Hepatitis A</td>
<td>1987</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>Hepatitis B</td>
<td>1984</td>
</tr>
<tr>
<td>Hepatitis C virus</td>
<td>Hepatitis C</td>
<td>1990</td>
</tr>
<tr>
<td>Herpes simplex virus, Type I</td>
<td>Cold Sores</td>
<td>1988</td>
</tr>
<tr>
<td>Human immunodeficiency virus (HIV-1)</td>
<td>Acquired immunodeficiency syndrome (AIDS)</td>
<td>1985</td>
</tr>
<tr>
<td>Human papillomavirus</td>
<td>Cervical cancer</td>
<td>1985</td>
</tr>
<tr>
<td>Human poliovirus</td>
<td>Poliomyelitis</td>
<td>1981</td>
</tr>
<tr>
<td>Human rhinovirus</td>
<td>Common cold</td>
<td>1984</td>
</tr>
<tr>
<td>Variola virus</td>
<td>Smallpox</td>
<td>1992</td>
</tr>
</tbody>
</table>

Using Genetic Engineering To Make Vaccines

Recombinant vaccines (selection)

<table>
<thead>
<tr>
<th>viruses</th>
<th>antigen</th>
<th>status</th>
</tr>
</thead>
<tbody>
<tr>
<td>hepatitis B</td>
<td>surface antigens</td>
<td>registered</td>
</tr>
<tr>
<td>Herpes simplex type 2</td>
<td>surface antigens</td>
<td>clinical studies</td>
</tr>
<tr>
<td>rabies vaccine</td>
<td>surface antigens</td>
<td>not registered</td>
</tr>
<tr>
<td>yellow fever virus</td>
<td>surface antigens</td>
<td>preclinical studies</td>
</tr>
<tr>
<td>AIDS virus</td>
<td>surface antigens</td>
<td>clinical studies</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>bacteria</th>
<th>antigen</th>
<th>status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pneumoniae</td>
<td>polysaccharide conjugate</td>
<td>registered</td>
</tr>
<tr>
<td>Clostridium tetani</td>
<td>tetanus toxin</td>
<td>not registered</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>surface antigens</td>
<td>clinical studies</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>parasites</th>
<th>antigen</th>
<th>status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium falciparum</td>
<td>(malaria)</td>
<td>clinical studies</td>
</tr>
<tr>
<td>Trypanosoma sp.</td>
<td>(sleeping sickness)</td>
<td>clinical studies</td>
</tr>
<tr>
<td>Schistosoma mansoni</td>
<td>(bilharziosis)</td>
<td>clinical studies</td>
</tr>
</tbody>
</table>

Vaccination by recombinant Vaccinia virus

- Gene coding for hepatitis B coat protein
- Recombinant Vaccinia virus injection into blood
- Vaccinia virus
- B-cells synthesize antibodies
- Antibody against Vaccinia virus
- Antibody against hepatitis-B coat protein

Immunization with virus coat protein or DNA

- Bioreactor process with recombinant yeast
- Injection into muscle
- Injection, oral, intranasal administration
- B-cells synthesize antibody
- Recombinant virus coat protein
- Plasmid with gene for virus coat protein
- In the event of infection, antibodies bind to virus and lead to its destruction by the immune system

Fermentation and recovery of recombinant hepatitis B vaccine

- Bioreactor
  - Recombinant S. cerevisiae expresses plasmid-coded rHBAg protein
- Recovery
  - By precipitation, diafiltration, chromatography
- rHBAg vaccine
- Complex quality control (absence of pathogens, allergens, etc.)
### Table 11.1 Human disease agents for which recombinant vaccines are currently being developed

<table>
<thead>
<tr>
<th>Pathogenic agent</th>
<th>Disease(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viruses</td>
<td>Chicken pox</td>
</tr>
<tr>
<td>Varicella-zoster virus</td>
<td>Infection in infants and immunocompromised patients</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>Hemorrhagic fever</td>
</tr>
<tr>
<td>Dengue virus</td>
<td>High fever, liver damage</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Long-term liver damage</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>Genital ulcers</td>
</tr>
<tr>
<td>Herpes simplex virus type 2</td>
<td>Acute respiratory disease</td>
</tr>
<tr>
<td>Influenza A and B viruses</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Japanese encephalitis virus</td>
<td>Inflammation of the upper respiratory tract</td>
</tr>
<tr>
<td>Parainfluenza virus</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>Upper and lower respiratory tract lesions</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>Acute infantile gastroenteritis</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Lesions of heart, kidney, and liver</td>
</tr>
<tr>
<td>Yellow fever virus</td>
<td>AIDS</td>
</tr>
<tr>
<td>Human immunodeficiency virus</td>
<td></td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td>Cholera</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>Diarrheal disease</td>
</tr>
<tr>
<td><em>E. coli</em> enterotoxin strains</td>
<td>Gonorrhea</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>Meningitis, septicemic conditions</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>Leprosy</td>
</tr>
<tr>
<td><em>Mycobacterium leprae</em></td>
<td>Meningitis</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>Whooping cough</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>Dysentery</td>
</tr>
<tr>
<td><em>Shigella</em> strains</td>
<td>Scarlet fever, rheumatic fever, throat infection</td>
</tr>
<tr>
<td><em>Streptococcus</em> group A</td>
<td>Sepsis, urogenital tract infection</td>
</tr>
<tr>
<td><em>Streptococcus</em> group B</td>
<td>Pneumonia, meningitis</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>Tetanus</td>
</tr>
<tr>
<td><em>Clostridium tetani</em></td>
<td>Tuberculosis</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Typhoid fever</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td></td>
</tr>
<tr>
<td><strong>Parasites</strong></td>
<td>River blindness</td>
</tr>
<tr>
<td><em>Onchocerca volvulus</em></td>
<td>Internal and external lesions</td>
</tr>
<tr>
<td><em>Leishmania</em> spp.</td>
<td>Malaria</td>
</tr>
<tr>
<td><em>Plasmodium</em> spp.</td>
<td>Schistosomiasis</td>
</tr>
<tr>
<td><em>Schistosoma mansoni</em></td>
<td>Sleeping sickness</td>
</tr>
<tr>
<td><em>Trypanosoma</em> spp.</td>
<td>Filariasis</td>
</tr>
<tr>
<td><em>Wuchereria bancrofti</em></td>
<td></td>
</tr>
</tbody>
</table>
Antibiotic resistance is a major problem.

RISING RESISTANCE

Many antibiotics are no longer effective against certain strains of bacteria, as these examples—collected from different hospitals in the late 1990s—show. One strain of Staphylococcus aureus found in Korea, for instance, is 98 percent resistant to penicillin (top left); another, found in the U.S., is 32 percent resistant to methicillin (bottom left). All these strains are not resistant to vancomycin, for now.

Staphylococcus aureus vs. penicillin: 98%
Staphylococcus aureus vs. methicillin: 32%
Enterococcus faecium vs. ciprofloxacin: 70%
Enterococcus faecium vs. ampicillin: 70%
Streptococcus pneumoniae vs. tetracycline: 10%
Streptococcus pneumoniae vs. penicillin: 37%

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<table>
<thead>
<tr>
<th>Antibiotics Synthesized in Microbes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 12.3</strong> Some of the most common microbially synthesized antibiotics</td>
</tr>
<tr>
<td>Amikacin sulfate</td>
</tr>
<tr>
<td>Amoxacillin</td>
</tr>
<tr>
<td>Ampicillin</td>
</tr>
<tr>
<td>Azithromycin</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
</tr>
<tr>
<td>Cefaclor</td>
</tr>
<tr>
<td>Cefixime</td>
</tr>
</tbody>
</table>
Using Genetic Engineering to Make Better/More Effective Antibiotics

Occurrence

<table>
<thead>
<tr>
<th>taxonomic group</th>
<th>relative number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycetes</td>
<td>50</td>
</tr>
<tr>
<td>other bacteria</td>
<td>10</td>
</tr>
<tr>
<td>fungi</td>
<td>20</td>
</tr>
<tr>
<td>lichens</td>
<td>1</td>
</tr>
<tr>
<td>algae</td>
<td>2</td>
</tr>
<tr>
<td>plants</td>
<td>15</td>
</tr>
<tr>
<td>animals</td>
<td>2</td>
</tr>
</tbody>
</table>

~25,000 compounds from nature

Systemic antibiotics (2001)

<table>
<thead>
<tr>
<th>type</th>
<th>value (billion US$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cephalosporins</td>
<td>6.7</td>
</tr>
<tr>
<td>penicillins</td>
<td>4.6</td>
</tr>
<tr>
<td>chinolones (synthetic)</td>
<td>4.6</td>
</tr>
<tr>
<td>macrolides</td>
<td>4.3</td>
</tr>
<tr>
<td>tetracyclines</td>
<td>0.7</td>
</tr>
<tr>
<td>aminoglycosides</td>
<td>0.6</td>
</tr>
<tr>
<td>peptide antibiotics, glycopeptides</td>
<td>0.5</td>
</tr>
<tr>
<td>other</td>
<td>2.2</td>
</tr>
<tr>
<td>total</td>
<td>24.2</td>
</tr>
</tbody>
</table>

Classification by chemical structure

1. carbohydrate antibiotics
   - aminoglycosides: streptomycin (medicine), kasugamycin (rice fungicide)

2. macrocyclic lactones
   - macrolides: erythromycin (medicine)
   - polyene antibiotics: polymixin (bacterial lysis)
   - ansamycines: rifampicin (cheese production), rifamycin (against tuberculosis)

3. chinones and related antibiotics
   - tetracyclines: tetracycline, chlorotetracycline (medicine, feed antibiotic)
   - anthracyclines: doxorubicin (cancer therapy)

4. amino acid and peptide antibiotics
   - amino acid derivatives: cyclosporin (organ transplantation)
   - β-lactam antibiotics: penicillin, cephalosporins (medicine)
   - peptide antibiotics: bacitracin (medicine), virginiamycin (feed antibiotic)
   - chromopeptides: actinomycin (cancer therapy), bleomycin (cancer therapy), vancomycin (medicine), avoparcine (cattle feed antibiotic)
   - glycopeptides: polyoxins, blasticidin S (fungicides for plant protection)

5. N-heterocyclic compounds
   - nucleoside antibiotics: monensin (chicken feed)

6. O-heterocyclic compounds
   - polyether antibiotics: cycloheximide (leaf fungicide)

7. allyclic compounds
   - cycloalkane derivatives: chloramphenicol (medicine)

8. aromatic antibiotics
   - benzene derivatives: griseofulvin (fungicide)

Antibiotics – point of attack

DNA replication

transcription

mRNA translation

70S ribosome

70S 50S

polyenes cell wall β-lactam antibiotics cell membrane 30S

β-lactam antibiotics

tetracycline

rifampicin

erythromycin

streptomycin

chloramphenicol

newly synthesized protein

24
Figure 12.9 Schematic representation of DNA transformation and selection of transformants of *Streptomyces* strains. The pink circles represent transformed cells, and the green circles represent nontransformed cells. PEG, polyethylene glycol.
Figure 5.16 Using Molecular Techniques to Identify Bacteria. Many molecular techniques are available for identifying bacteria. (a) RFLP is one such technique. For some pathogens, isolated DNA (which may come from a clinical sample such as blood or saliva) can be subjected to restriction enzyme digestion and separation by agarose gel electrophoresis. Banding patterns of DNA fragments can be compared to reference strains of known bacteria to allow for a positive identification. In this example, bacterial DNA isolated from the clinical sample matches *P. aeruginosa*. (b) PCR can also be used for bacterial identification. PCR has the advantage of being much more sensitive than RFLP analysis; therefore, only small amounts of clinical samples and small amounts of DNA are required. The sensitivity of PCR also makes it possible to identify small amounts of DNA from just a few cells allowing for early treatment of an infection. (c) DNA sequencing strategies are also commonly used for microbial identification.

2.7. Food Poisoning Bacteria

*B. anthracis*
Enzymes as additives in industry

<table>
<thead>
<tr>
<th>application</th>
<th>enzyme type</th>
<th>organisms (examples)</th>
<th>market size (% of total)</th>
<th>economic advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>detergents</td>
<td>proteases, cellulases, lipases</td>
<td>Bacillus licheniformis, Aspergillus niger, Trichoderma reesei</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>starch hydrolysis</td>
<td>α-amylase</td>
<td>Bacillus amylobacteriacei</td>
<td>5</td>
<td>3, 4</td>
</tr>
<tr>
<td>glucose isomerization</td>
<td>glucose isomerase</td>
<td>Streptomyces venezuelae</td>
<td>7</td>
<td>1, 3</td>
</tr>
<tr>
<td>beer-brewing</td>
<td>amylase</td>
<td>Bacillus subtilis</td>
<td>3</td>
<td>3, 4</td>
</tr>
<tr>
<td>fruit processing, wine</td>
<td>cellulases, hemicellulases, pectinases</td>
<td>Aspergillus niger</td>
<td>5</td>
<td>3, 4, 5, 6</td>
</tr>
<tr>
<td>flour, bakery goods</td>
<td>α-amylase, proteases</td>
<td>Aspergillus oryzae</td>
<td>8</td>
<td>1, 3</td>
</tr>
<tr>
<td>cheese manufacture, aroma</td>
<td>proteases, chymosin, lipases</td>
<td>animal rennin, Rhizomucor miehei, Saccharomyces cerevisiae</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>sludge and animal feed</td>
<td>phytases</td>
<td>Aspergillus niger</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>paper and textiles</td>
<td>α-amylase, lipase</td>
<td>Bacillus Humicola</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>leather treatment</td>
<td>proteases</td>
<td>Aspergillus oryzae</td>
<td>10</td>
<td>1, 7</td>
</tr>
</tbody>
</table>

Quantities for different applications:

- High-fructose syrups
- Bakery goods
- Cheese
- Leather
- Fruits and wine
- Starch hydrolysis

<table>
<thead>
<tr>
<th>process/application</th>
<th>enzyme cost per unit quantity (US $)</th>
</tr>
</thead>
<tbody>
<tr>
<td>starch liquefaction</td>
<td>ca. $2 per t starch</td>
</tr>
<tr>
<td>glucose from starch</td>
<td>$3.5 per t starch</td>
</tr>
<tr>
<td>isomerization of glucose</td>
<td>$6 per t starch</td>
</tr>
<tr>
<td>HFS in USA</td>
<td>$6 - 7 per t starch</td>
</tr>
<tr>
<td>ethanol</td>
<td>$1 per t starch</td>
</tr>
<tr>
<td>beer</td>
<td>$0.1 per 100 L</td>
</tr>
<tr>
<td>bakery goods USA</td>
<td>$0.1 per 100 kg flour</td>
</tr>
<tr>
<td>bakery goods EU</td>
<td>$0.1 - 0.5 per 100 kg flour</td>
</tr>
<tr>
<td>fruit juice</td>
<td>$0.1 - 0.5 per 100 L juice</td>
</tr>
<tr>
<td>wine</td>
<td>$0.1 - 0.5 per 100 L wine</td>
</tr>
<tr>
<td>stabilization of fruit</td>
<td>$0.3 - 0.8 per 1000 L</td>
</tr>
<tr>
<td>lemonade by glucose oxidase</td>
<td>$0.05 per 100 mL milk</td>
</tr>
<tr>
<td>cheese manufacture</td>
<td>$0.05 per kg detergent</td>
</tr>
<tr>
<td>detergents</td>
<td>$1.2 - 3 per t skin</td>
</tr>
<tr>
<td>leather tanning</td>
<td></td>
</tr>
</tbody>
</table>

Important goals in application technology:

1. Higher product quality
2. Improved taste
3. Better yields
4. Reduced process costs
5. Better filtration
6. Better conservation
7. Improved working conditions, reduced environmental load
Recombinant chymosin is used to make cheese.

### Composition of milk

<table>
<thead>
<tr>
<th>Component</th>
<th>Milk (%)</th>
<th>Whey (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>88</td>
<td>94</td>
</tr>
<tr>
<td>Fat</td>
<td>3-4</td>
<td>0.5</td>
</tr>
<tr>
<td>Protein</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>Casein</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
<td>4.8</td>
</tr>
</tbody>
</table>

### Processing of milk

- Seur milk products: starter cultures → milk: lactase, acid, Ca²⁺, chymosin → reduced-lactose milk products
- Cheese curd: lactase, membrane separation: whey, lactose and whey protein
- Soft cheese: lipases, proteases, other enzymes, starter cultures
- Hard cheese: lactose syrup

### Manufacture of chymosin

**Native**
- Stomachs of young animals: cutting, activation at pH < 5
- Extraction: salt water, 14d
- Purification: ultrafiltration, standardization
- 200 U/kg stomach

**Recombinant**
- Preculture: high-yield mutants of *Mucor miehei* or *M. pusillus*
- Bioreactor: dextrose syrup, soy meal, 30°C, 72h
- Purification: separation of mycelium, reverse osmosis, precipitation
- 50000 U/m² in 72h

**Recombinant microbial**
- Escherichia coli
- Bioreactor: maltodextrins, 37°C, 36h
- Purification: isolation of inclusion bodies, Triton-X100/EDTA, urea-alcaline extract, ion-exchange chromatography, acid treatment
- 200000 U/m² in 36h

Chymosin acts on milk protein to coagulate milk into cheese!

Is cheese a food?
Chymosin in Cheese Making

1. 80-90% of cheeses are made with Recombinant Chymosin
2. Approved for use in cheesemaking by FDA
3. Not different from non-recombinant Chymosin - i.e. GRAS - Generally Regarded as Safe & not labeling needed - because not an additive & not different from non-recombinant chymosin!

Is Cheese Made Using a GMO?

Industry adds claim that Recombinant Chymosin is "Kosher" & "Vegetarian"
PROTEIN ENGINEERING - EVOLUTION IN A TEST TUBE!!

Evolution

Ancestral species

Species 1

Species 2

Species 3

Species 4

DNA shuffling in vitro

Hybrid genes

Novel enzymes and other proteins!

Fig. 14.14 Schematic representation of gene shuffling.

Making enzymes not in "nature" that work more efficiently using recombinant DNA!

e.g., more effective chymosin!
# Useful Bacterial Metabolites that Can Be Engineered

## Table 5.1: Examples of Primary and Secondary Metabolites Produced by Fermentation

<table>
<thead>
<tr>
<th>Primary Metabolites</th>
<th>Secondary Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>Antibiotics</td>
</tr>
<tr>
<td>Vitamins</td>
<td>Pigments</td>
</tr>
<tr>
<td>Nucleotides</td>
<td>Toxins</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Alkaloids</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Many active pharmacological compounds (e.g., the immunosuppressor cyclosporin, hypotensive compound dopasatin)</td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
</tr>
<tr>
<td>Acryl acid</td>
<td></td>
</tr>
<tr>
<td>Butanol</td>
<td></td>
</tr>
<tr>
<td>2,3-Butanediol</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td>Formic acid</td>
<td></td>
</tr>
<tr>
<td>Fumaric acid</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
</tr>
<tr>
<td>Glycolic acid</td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td></td>
</tr>
<tr>
<td>Methyethyl ketone</td>
<td></td>
</tr>
<tr>
<td>Oxalic acid</td>
<td></td>
</tr>
<tr>
<td>Propylene glycol</td>
<td></td>
</tr>
<tr>
<td>Succinic acid</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organic Chemical</th>
<th>Microbial Sources</th>
<th>Selected Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Acetobacter</td>
<td>Industrial solvent and intermediate for many organic chemicals, food acidulant</td>
</tr>
<tr>
<td>Acetone</td>
<td>Clostridium</td>
<td>Industrial solvent and intermediate for many organic chemicals</td>
</tr>
<tr>
<td>Acryl acid</td>
<td>Bacillus</td>
<td>Industrial intermediate for plastics</td>
</tr>
<tr>
<td>Butanol</td>
<td>Clostridium</td>
<td>Industrial solvent and intermediate for many organic chemicals</td>
</tr>
<tr>
<td>2,3-Butanediol</td>
<td>A. propionicus;</td>
<td>Intermediate for synthetic rubber manufacture, plastics and antifreeze</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Saccharomyces</td>
<td>Industrial solvent, intermediate for vinegar, esters and ethers, beverages</td>
</tr>
<tr>
<td>Formic acid</td>
<td>Aspergillus</td>
<td>Textile dyeing, leather treatment, electrophotating, rubber manufacture</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>Rhizopus</td>
<td>Intermediate for synthetic resins, dyeing, acidulant, antioxidant</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Saccharomyces</td>
<td>Solvent, plasticizer, sweetener, explosives manufacture, printing, cosmetics, soaps, antifreeze</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>Aspergillus</td>
<td>Textile processing, pH control, adhesives, cleaners</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Clostridum</td>
<td>Industrial solvent, cosmetic preparations, antifreeze, inks</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>Lactobacillus;</td>
<td>Food acidulant, dyeing, intermediate for lactates, leather treatment</td>
</tr>
<tr>
<td>Methyethyl ketone</td>
<td>Chlamydomonas</td>
<td>Industrial solvent, intermediate for explosives and synthetic resins</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>Aspergillus</td>
<td>Printing and dyeing, bleaching agent, cleanser, reducing agent</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>Bacillus</td>
<td>Antiblueze, solvent, synthetic resin manufacture, mold inhibitor</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>Rhizopus</td>
<td>Manufacture of lacquers, dyes and esters for perfume</td>
</tr>
</tbody>
</table>
**Bacterial Metabolic Pathways**

Can be engineered to optimize production of novel industrial products.

---

Fig. 6.5 The formation of commercially useful metabolic end-products. Note that pyridine nucleotide cofactors are reduced during the conversion of sugars to pyruvate and subsequently oxidized by further metabolism of pyruvate.

---

Fig. 6.4 The different classes of low-molecular-weight compounds synthesized by microorganisms.
MICROBES CAN BE ENGINEERED TO PRODUCE IMPORTANT MOLECULES THAT WERE MADE PREVIOUSLY BY CHEMICAL REACTIONS.

\[ \text{Vitamin C synthesis} \]

**Chemical**

(a)  
\[ \text{d-Glucose} \xrightarrow{\text{Chemical}} \text{d-Sorbitol} \xrightarrow{\text{Acetobacter suboxydans}} \text{l-Sorbose} \]

(b)  
\[ \text{Ascorbic acid} \xrightarrow{\text{Chemical}} \text{2-Keto-L-gulonic acid} \xrightarrow{\text{Chemical}} \text{Diacetone 2-keto-L-gulonic acid} \]

**Biologically-based**

Fig. 6.12 Simplified route to vitamin C (ascorbic acid) developed by cloning in Erwinia the Corynebacterium gene for 2,5-diketogluconic acid reductase. (a) Classical route to vitamin C. (b) The simplified route to 2-ketogulonic acid, the immediate precursor of vitamin C.
**Figure 12.8** Indigo biosynthesis from tryptophan in genetically engineered *E. coli*. Tryptophanase is an *E. coli* enzyme. In pathway A, the naphthalene dioxygenase is derived from the NAH plasmid; in pathway B, the xylene oxidase is from the TOL plasmid. *E. coli* transformants that synthesize indigo contain either pathway A or B but not both pathways.

A $200$ M/yr industry.

Indigo previously obtained from plants!
### TABLE 9.1 TWENTY OF THE MOST COMMON CHEMICAL POLLUTANTS IN THE ENVIRONMENT

<table>
<thead>
<tr>
<th>Chemical Pollutant</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>Petroleum products used to make plastics, nylon, resins, rubber, detergents, and many other materials</td>
</tr>
<tr>
<td>Creosote</td>
<td>Wood preservative to prevent rotting</td>
</tr>
<tr>
<td>Cyanide</td>
<td>Mining processes and manufacturing of plastics and metals</td>
</tr>
<tr>
<td>Dioxin</td>
<td>Pulp and paper bleaching, waste incineration, and chemical manufacturing processes</td>
</tr>
<tr>
<td>Methyl t-butyl ether (MTBE)</td>
<td>Fuel additive, automobile exhaust, boat engines, leaking gasoline tanks</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Product of crude oil and petroleum</td>
</tr>
<tr>
<td>Nitriles</td>
<td>Rubber compounds, plastics, and oils</td>
</tr>
<tr>
<td>Perchloroethylene</td>
<td>Dry cleaning agent</td>
</tr>
<tr>
<td>Pesticides (atrazine, carbamates, chlordeane, DDT) and herbicides</td>
<td>Chemicals used to kill insects (pesticides) and weeds (herbicides)</td>
</tr>
<tr>
<td>Phenol and related compounds (chlorophenols)</td>
<td>Wood preservatives, paints, glues, textiles</td>
</tr>
<tr>
<td>Polychlorinated biphenyls (PCBs)</td>
<td>Electrical transistors, cooling and insulating systems</td>
</tr>
<tr>
<td>Polycyclic aromatic hydrocarbons (PAHs) and polychlorinated hydrocarbons</td>
<td>Incineration of wastes, automobile exhaust, oil refineries, and leaking oil from cars</td>
</tr>
<tr>
<td>Polyvinylchloride</td>
<td>Plastic manufacturing</td>
</tr>
<tr>
<td>Radioactive compounds</td>
<td>Research and medical institutions and nuclear power plants</td>
</tr>
<tr>
<td>Surfactants (detergents)</td>
<td>Manufacturing of paints, textiles, concrete, paper</td>
</tr>
<tr>
<td>Synthetic estrogens (ethinyl estradiol)</td>
<td>Female hormone (estrogen)-related compounds created by a variety of industrial manufacturing processes</td>
</tr>
<tr>
<td>Tetrachloroethylene and trichloroethylene</td>
<td>Dry cleaning chemicals and degreasing agents</td>
</tr>
<tr>
<td>Toluene</td>
<td>Petroleum component present in adhesives, inks, paints, cleaners, and glues</td>
</tr>
<tr>
<td>Trace metals (arsenic, cadmium, chromium, copper, lead, mercury, silver)</td>
<td>Car batteries and metal manufacturing processes</td>
</tr>
<tr>
<td>Trinitrotoluene (TNT)</td>
<td>Explosive used in building and construction industries</td>
</tr>
</tbody>
</table>
Table 13.1  *Pseudomonas* plasmids, their degradative pathways, and their sizes

<table>
<thead>
<tr>
<th>Name of plasmid</th>
<th>Compound(s) degraded</th>
<th>Plasmid size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>Salicylate</td>
<td>60</td>
</tr>
<tr>
<td>SAL</td>
<td>Salicylate</td>
<td>72</td>
</tr>
<tr>
<td>SAL</td>
<td>Salicylate</td>
<td>83</td>
</tr>
<tr>
<td>TOL</td>
<td>Xylene and toluene</td>
<td>113</td>
</tr>
<tr>
<td>pJP1</td>
<td>2,4-D</td>
<td>87</td>
</tr>
<tr>
<td>pJP2</td>
<td>2,4-D</td>
<td>54</td>
</tr>
<tr>
<td>pJP3</td>
<td>2,4-D</td>
<td>78</td>
</tr>
<tr>
<td>CAM</td>
<td>Camphor</td>
<td>225</td>
</tr>
<tr>
<td>XYL</td>
<td>Xylene</td>
<td>15</td>
</tr>
<tr>
<td>pAC31</td>
<td>3,5-Dichlorobenzoate</td>
<td>108</td>
</tr>
<tr>
<td>pAC25</td>
<td>3-Chlorobenzoate</td>
<td>102</td>
</tr>
<tr>
<td>pWWO</td>
<td>Xylene and toluene</td>
<td>176</td>
</tr>
<tr>
<td>NAH</td>
<td>Naphthalene</td>
<td>69</td>
</tr>
<tr>
<td>XYL-K</td>
<td>Xylene and toluene</td>
<td>135</td>
</tr>
</tbody>
</table>


Plasmids with the same name encode a similar degradative pathway even though they have different sizes and were described in different laboratories. 2,4-D, 2,4-dichlorophenoxyacetic acid.
Bacteria can be engineered to degrade several different "toxic" compounds.

Figure 13.5 Schematic representation of the development of a bacterial strain that can degrade camphor, octane, xylene, and naphthalene. Strain A, which contains a CAM (camphor-degrading) plasmid, is mated with strain B, which carries an OCT (octane-degrading) plasmid. Following plasmid transfer and homologous recombination between the two plasmids, strain E carries a CAM and OCT biodegradative fusion plasmid. Strain C, which contains a XYL (xylene-degrading) plasmid, is mated with strain D, which contains a NAH (naphthalene-degrading) plasmid, to form strain F, which carries both of these plasmids. Finally, strain E and strain F are mated to yield strain G, which carries the CAM/OCT fusion plasmid, the XYL plasmid, and the NAH plasmid.

Chakrabarty vs Patent 4,253,444 1981

Genetically engineered microorganisms are "inventions."

Life can be patented!
Figure 12.15 Enzymatic biodegradation of cellulose. Cellulose hydrolysis begins with the cleavage of β-1,4-linkages within the accessible amorphous regions of the cellulose chains by endoglucanase(s). This reaction is followed by the removal of oligosaccharides from the reducing ends of the partially cleaved cellulose chains by exoglucanase(s) and cellbiohydrodrolase(s). The degradation of cellulose is completed when the cellbiose and cellotriose are converted to glucose by β-glucosidase.

Agriculture, Timber Processing, Human Activities:
- e.g., plants left after harvest, animal manure with grass, municipal waste paper, rotten lettuce, hay, etc.

Waste containing cellulose

Energy for bacteria

Green waste!
Engineering *Deinococcus radiodurans* for metal remediation in radioactive mixed waste environments

Hassan Brim¹, Sara C. McFarlan², James K. Fredrickson³, Kenneth W. Minton¹, Min Zhai¹, Lawrence P. Wackett² & Michael J. Daly¹

1. Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814.
2. Department of Biochemistry, Biological Process Technology Institute and Center for Biodegradation Research and Informatics, Gortner Laboratory, University of Minnesota, St. Paul, MN 55108.
3. Pacific Northwest National Laboratory, Richland, WA 99352.
Correspondence should be addressed to M J Daly. e-mail: mdaly@usuhs.mil

We have developed a radiation resistant bacterium for the treatment of mixed radioactive wastes containing ionic mercury. The high cost of remediating radioactive waste sites from nuclear weapons production has stimulated the development of bioremediation strategies using *Deinococcus radiodurans*, the most radiation resistant organism known. As a frequent constituent of these sites is the highly toxic ionic mercury (Hg) (II), we have generated several *D. radiodurans* strains expressing the cloned Hg (II) resistance gene (*merA*) from *Escherichia coli* strain BL308. We designed four different expression vectors for this purpose, and compared the relative advantages of each. The strains

\[ \text{Hg}^{II} \rightarrow \text{Hg}^{0} \rightarrow \text{Vapor} \]

Metal Chelate in Safe
Aerobic degradation of 2,4,6-trinitrotoluene by Enterobacter cloaca PB2 and by pentaerythritol tetroxide reductase.

French CE, Nicklin S, Bruce NC.

Institute of Biotechnology, University of Cambridge, Cambridge CB2 1QT, United Kingdom.

Enterobacter cloaca PB2 was originally isolated on the basis of its ability to utilize nitrate esters, such as pentaerythritol tetroxide (PETN) and glycerol trinitrate, as the sole nitrogen source for growth. The enzyme responsible is an NADPH-dependent reductase designated PETN reductase. E. cloaca PB2 was found to be capable of slow aerobic growth with 2,4,6-trinitrotoluene (TNT) as the sole nitrogen source. Dinitrotoluene was not produced and could not be used as nitrogen sources. Purified PETN reductase was found to reduce TNT to its hydride-Meisenheimer complex, which was further reduced to the dihydride-Meisenheimer complex. Purified PETN reductase and recombinant Escherichia coli expressing PETN reductase were able to liberate nitrogen as nitrite from TNT. The ability to remove nitrogen from TNT suggests that PB2 or recombinant organisms expressing PETN reductase may be useful for bioremediation of TNT-contaminated soil and water.
Biodegradation of explosives by transgenic plants expressing pentaerythritol tetranitrate reductase

Christopher E. French¹,³, Susan J. Rosser¹, Gareth J. Davies¹, Stephen Nicklin² & Neil C. Bruce¹

1. Institute of Biotechnology, University of Cambridge, Tennis Court Rd., Cambridge CB2 1QT, UK.
Correspondence should be addressed to N C Bruce. e-mail: n.brace@biotech.cam.ac.uk

Plants offer many advantages over bacteria as agents for bioremediation; however, they typically lack the degradative capabilities of specially selected bacterial strains. Transgenic plants expressing microbial degradative enzymes could combine the advantages of both systems. To investigate this possibility in the context of bioremediation of explosive residues, we generated transgenic tobacco plants expressing pentaerythritol tetranitrate reductase, an enzyme derived from an explosive-degrading bacterium that enables degradation of nitrate ester and nitroaromatic explosives. Seeds from transgenic plants were able to germinate and grow in the presence of 1 mM glycerol trinitrate (GTN) or 0.05 mM trinitrotoluene, at concentrations that inhibited
SESSION I. STATE OF THE ART: CASE HISTORIES

Ecology of *Pseudomonas syringae* Relevant to the Field Use of Ice− Deletion Mutants Constructed In Vitro for Plant Frost Control

S.E. LINDOW

Department of Plant Pathology, University of California, Berkeley, California 94720

How everything that can go wrong, goes wrong in the face of elegant, logical science!
<table>
<thead>
<tr>
<th>Rank</th>
<th>Crops in California&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Area planted (106 hectares)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alfalfa</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>Cotton lint, all</td>
<td>0.34</td>
</tr>
<tr>
<td>3</td>
<td>Grapes, all</td>
<td>0.32</td>
</tr>
<tr>
<td>4</td>
<td>Rice</td>
<td>0.20</td>
</tr>
<tr>
<td>5</td>
<td>Almond (shelled)</td>
<td>0.19</td>
</tr>
<tr>
<td>6</td>
<td>Wheat, all</td>
<td>0.18</td>
</tr>
<tr>
<td>7</td>
<td>Tomato, all</td>
<td>0.15</td>
</tr>
<tr>
<td>8</td>
<td>Lettuce, all</td>
<td>0.09</td>
</tr>
<tr>
<td>9</td>
<td>Maize, grain</td>
<td>0.082</td>
</tr>
<tr>
<td>10</td>
<td>Oranges, all</td>
<td>0.081</td>
</tr>
<tr>
<td>11</td>
<td>Walnuts</td>
<td>0.077</td>
</tr>
<tr>
<td>12</td>
<td>Beans, dry</td>
<td>0.053</td>
</tr>
<tr>
<td>13</td>
<td>Barley</td>
<td>0.05</td>
</tr>
<tr>
<td>14</td>
<td>Broccoli</td>
<td>0.05</td>
</tr>
<tr>
<td>15</td>
<td>Sugar beets</td>
<td>0.043</td>
</tr>
<tr>
<td>16</td>
<td>Carrots</td>
<td>0.037</td>
</tr>
<tr>
<td>17</td>
<td>Prunes, dry</td>
<td>0.034</td>
</tr>
<tr>
<td>18</td>
<td>Pistachios</td>
<td>0.029</td>
</tr>
<tr>
<td>19</td>
<td>Sunflower</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Canola</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rank</th>
<th>Crops in the U.S.&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Area planted (106 hectares)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maize (including sweet corn)</td>
<td>32.5</td>
</tr>
<tr>
<td>2</td>
<td>Soybean</td>
<td>30.1</td>
</tr>
<tr>
<td>3</td>
<td>Wheat</td>
<td>25.3</td>
</tr>
<tr>
<td>4</td>
<td>Alfalfa</td>
<td>9.3</td>
</tr>
<tr>
<td>5</td>
<td>Cotton</td>
<td>6.3</td>
</tr>
<tr>
<td>6</td>
<td>Sorghum</td>
<td>4.3</td>
</tr>
<tr>
<td>7</td>
<td>Barley</td>
<td>2.4</td>
</tr>
<tr>
<td>8</td>
<td>Oats</td>
<td>1.8</td>
</tr>
<tr>
<td>9</td>
<td>Rice</td>
<td>1.2</td>
</tr>
<tr>
<td>10</td>
<td>Sunflower</td>
<td>1.1</td>
</tr>
<tr>
<td>11</td>
<td>Beans, dry and snap</td>
<td>0.8</td>
</tr>
<tr>
<td>12</td>
<td>Rapeseed (including canola)</td>
<td>0.6</td>
</tr>
<tr>
<td>13</td>
<td>Beets, sugar and table</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>Groundnuts (peanuts)</td>
<td>0.5</td>
</tr>
<tr>
<td>15</td>
<td>Potatoes</td>
<td>0.5</td>
</tr>
<tr>
<td>16</td>
<td>Rye</td>
<td>0.5</td>
</tr>
<tr>
<td>17</td>
<td>Sugar cane</td>
<td>0.4</td>
</tr>
<tr>
<td>18</td>
<td>Grapes</td>
<td>0.4</td>
</tr>
<tr>
<td>19</td>
<td>Oranges</td>
<td>0.3</td>
</tr>
<tr>
<td>20</td>
<td>Flaxseed</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimated area of production in California for 2000 (California Department of Food and Agriculture Resource Directory)

<sup>b</sup> Estimated area of production in U.S. for 2000 (USDA)

Table 2. The top 20 crops grown in California and the United States, and the transgenic crops no longer regulated by USDA (in boldface)
CITRUS PRODUCTION

TOTAL USA 1998 - $2.6 Billion
Export Value - $650 Million Exported
Total Fruit Exports - $1.6 Billion Supporting $3.2 Billion in Economic Activity & 33,000 jobs!

CALIFORNIA CITRUS

TOTAL FARM VALUE 2001 - $30 billion
Citrus Value - $1.5 billion
Valencia Statistics

Year


Production (Cartons)
Avg. Cvr. Rt

CITRUS LOSS DUE TO FREEZING

FROST
ICE FORMATION

Water cooling and freezing

2° C
0° C
-3° C
-8° C

---
Snomax (ICE+ bacteria)
---
No Snomax

FREEZING requires supercooling + ice crystal nucleation.
Pseudomonas Ice+ bacteria can do this.

Note: ICE+ bacteria nucleate supercooled water at a higher temperature to cause freezing!
Pseudomonas syringae

Gram-negative heterotrophic rods with polar flagella. Very common form of soil bacteria; also contain many important plant pathogens.

Genome = 6.7 Mbp

Colonizes / Plants - lives on leaves
Some strains bacteria - Some not
Frost-sensitive plants cannot tolerate ice crystal formation within their tissues. Ice crystals within sensitive plant tissues propagate rapidly, both intercellularly and intracellularly, causing mechanical breakdown of plant tissue and subsequent death. Many liquids, including water, do not invariably freeze at the melting-point of the solid phase. These liquids can be supercooled below the melting-point of the solid phase, e.g. water can be supercooled to $-10^\circ\text{C}$ to $-20^\circ\text{C}$. The water–ice phase transition requires the presence of a catalyst or ice nucleus. Plants do not have intrinsic ice nuclei active at temperatures above $-5^\circ\text{C}$ but certain bacterial species can act as ice nuclei and thus have a primary role in limiting supercooling and inciting frost damage to frost-sensitive plants. The commonest ice nucleation-active bacteria isolated from plants are *Pseudomonas syringae* and *Erwinia herbicola*. They can initiate ice formation at temperatures of $-1.5^\circ\text{C}$ to $-5^\circ\text{C}$, most probably by means of an outer membrane component. The gene(s) for ice nucleation have been cloned and are under active study.

Ice nucleation-active bacteria are present in large numbers in all temperate regions of the world and may be important in initiating rain and snow. Currently these bacteria are used to facilitate the formation of ‘artificial’ snow on ski slopes. Water containing *P. syringae* is sprayed through a fine nozzle on to a fan and the expansion-induced cooling produces snow.
P06620

ICN nucleation protein.

P. syringae pv. syringae

Green, R.L. and Warren, G.J.

Physical and functional repetition in a bacterial ice nucleation gene


Localization of ice nucleation activity and the iceC gene product in P. syringae and Escherichia coli


A model of the three-dimensional structure of ice nucleation proteins


3D-STRUCTURE MODELING OF 490-535.

This SWISS-PROT entry is copyright. It is produced through a collaboration between the Swiss Institute of Bioinformatics and the EMBL outstation – the European Bioinformatics Institute. The original entry is available from http://www.expasy.ch/sprot and http://www.ebi.ac.uk/sprot

Ice nucleation proteins enable bacteria to nucleate crystallization in supercooled water.

[FUNCTION] Ice nucleation proteins enable bacteria to nucleate crystallization in supercooled water.

[SUBCELLULAR LOCATION] Outer membrane.

[DOMAIN] Contains 122 imperfect repeats of the consensus octapeptide A-G-Y-G-S-S-L-T; further on a 16-residue and a regional 48-residue periodicity is superimposed.

[MISCELLANEOUS] A structural model is suggested in which the ice nucleation protein displays a symmetry related to that of ice.

[SIMILARITY] Belongs to the bacterial ice nucleation protein family.
Creation of Ice- Pseudomonas

Wild type

Ice nucleation protein

Ice+

Deletion mutant using genetic engineering

Ice- mutant

Ice- nucleation protein

No ice nucleation

Nucleates Ice formation on

Death

Survives lower temperature!
GROWTH OF WILD-TYPE AND DELETED PSEUDOMONAS STRAINS

FIG. 2. Population size of *P. syringae* TLP2 (○-○) and TLP2del1 (■-■) as a function of time on wet potato leaves. Potato leaves were inoculated with an aqueous suspension of ca. 10⁴ CFU of each bacterial strain per ml and placed in a mist chamber at 24°C. Leaves were harvested and bacterial populations were determined after the times shown on the abscissa by dilution plating of leaf washings onto King’s medium B plus rifampin. The vertical bars represent the standard error of the mean population size determined from four samples at each sampling time.

FIG. 4. Survival of Ice⁺ parental strains (○-○) and Ice⁻ deletion mutants (●-●) of *P. syringae* TLP2 (A) and Cit7 (B) during successive freeze-thaw cycles. Bacterial strains were suspended in tubes containing 100 mM potassium phosphate buffer (pH 7.0) containing 0.1% peptone at a concentration of ca. 3,000 to 6,000 CFU/ml. Tubes were cooled to −5°C for 15 min and then warmed to 25°C for 5 min to melt the frozen suspensions. Tubes containing Ice⁻ bacterial strains were inoculated with ice by touching their contents 3 min after transfer to a −5°C bath with a sterile needle cooled to −80°C. Tubes containing parental strains (□-□) or Ice⁻ deletion mutants (■-■) were also inoculated at 25°C for the duration of the experiment. A 0.1-ml sample of bacterial suspension was removed during successive melting cycles (or at an equivalent time for unfrozen control suspension) and plated onto King’s medium B plus rifampin. Points shown are the mean number of colonies recovered from two replications at each time of collection.

NO SIGNIFICANT GROWTH OR SURVIVAL DIFFERENCES
Hypothesis: Releasing ICE® Pseudomonas on crops should protect them from freezing!

SAVE CROPS x BILLIONS of DOLLARS of CROP LOSS!

Players:
Steve Linford, UC Berkeley
Advanced Genetic Sciences (AGS)
USDA
EPA
NIH RAC
Foundation for Economic Trends - Jeremy Rifkin et al.

Start: 1983 (33 years ago!)
Finish: 1987
Deliberate Release of GMOs

Despite its initial prohibition, by 1982 it became clear that the RAC would have to cope with requests for open-field testing of GMOs, i.e., for their deliberate release into the environment. Uncharacteristically, neither guidelines nor protocols that advised applicants what information should be included in their submissions had been prepared. This initial reluctance to establish definitive regulations was due to a widely held belief among many molecular biologists that GMOs were not significantly different from their nonengineered progenitors. And, if a difference was present, it was thought that it would be readily detected by conventional biological testing.

Three applications for field trials of GMOs were received by the RAC in 1982. Two dealt with genetically modified plants, viz., corn and tobacco.

The third proposal was concerned with testing a genetically modified strain of the microorganism Pseudomonas syringae to determine if it could limit the extent of frost damage to plants. This particular submission became part of the landmark case for the development of regulatory procedures for the release of GMOs into the environment.

Ice-Minus P. syringae

The genetic engineering portion of the P. syringae proposal involved removing a gene that codes for an ice nucleation protein from the organism and then testing whether the modified “ice-minus” strain, when sprayed onto the leaves of plants, could prevent frost damage. Under natural conditions, wild-type “ice-plus” P. syringae, which is usually found on the surface of plant leaves, secretes a protein that at low temperatures causes the formation of ice crystals, which, in turn, causes frost damage to the plant.

The rationale for the deletion of the gene encoding the ice nucleation protein was that if a strain that lacked this protein were sprayed onto leaves before they became colonized with the wild-type strain, it might lower the temperature at which ice formation would occur, thereby preventing the leaves from being damaged by bacterially induced ice crystals. There is a significant economic incentive for such a novel treatment, because in the United States crop losses due to frost damage exceed a billion dollars per year.

In response to each of the requests for field testing of a GMO, the RAC followed, more or less, the procedures it had established for handling the regulation of recombinant DNA experimentation in the laboratory:

1. The submissions were announced in the Federal Register.
2. Information was sent to 3,000 interested persons.
3. A panel of experts reviewed the proposals.
4. A public meeting was called for discussion of each proposal.
5. At the same time the RAC was reviewing the proposals, the U.S. Department of Agriculture (USDA) also reviewed them.

After careful consideration, both the USDA and the RAC approved the “ice-nucleation gene deletion” proposal. In 1983, the director of NIH gave final endorsement to the RAC decision. On the same day that permission was granted to proceed with the field trial, a lawsuit filed to block the test was filed by an organization called the Foundation on Economic Trends, which is headed by Jeremy Rifkin, who strongly opposes all forms of genetic engineering. The lawsuit was upheld, with the judge noting that the RAC had not carried out a proper hearing in accordance with U.S. statutes

and, more important, that it had failed to request an environmental impact statement.

This legal decision dramatically demonstrated that, notwithstanding the scientific opinion of the RAC and its experts, the existing regulatory system for GMO field testing was inadequate. A prevalent opinion outside the confines of the RAC was that the release of a genetically modified organism into the environment could have far-reaching effects because living microorganisms proliferate, persist, disperse, and sometimes transfer their DNA to other microorganisms. Some critics of the release of GMOs to the environment believed that, after its introduction into the environment, an engineered organism could displace an existing, important species from its ecological niche and as a result cause severe environmental damage. In addition, some opponents of release believed that genes could be transferred from an introduced GMO to indigenous strains, thereby creating, albeit inadvertently, an ecologically dangerous organism. Although these points of view presented worst-case, adverse-effect scenarios that might be exceedingly unlikely, it was essential that the regulatory protocol for field testing include a thorough assessment of the potential risk that an introduced organism might have on the environment.

The responsibility for assessing the initial submissions for the deliberate release of GMOs in the United States resided with the U.S. Environmental Protection Agency (EPA) and the USDA. The NIH drew up an initial set of criteria for field tests with GMOs, but it relinquished its authority in this area to these other agencies.

The EPA decided to use two applications, both dealing with ice nucleation-defective bacteria, as prototype cases for developing an assessment process for the field testing of GMOs. Each proposal went through a series of reviews, which included appraisals of the environmental, ethical, ecological effects, and human health consequences of the test as well as product analysis, by the following groups:

- The Office of Pesticide Program Review of the EPA
- The Toxic Substances, Research and Development Policy Planning, and Evaluation Committees of the EPA
- The General Counsel of the EPA
- The USDA, FDA, and NIH
- A Science Advisory Panel that consisted of a microbiologist, a plant pathologist, and a community ecologist
- Open public meetings
- Various state agencies, which in this instance included the California Department of Agriculture

It was not envisioned that this elaborate, time-consuming, and often redundant process would become the routine mechanism for approving field testing of GMOs; rather, it was assumed that, with experience, the system would be trimmed without loss of effective assessment of the potential hazards of each trial. After what was thought to be a very thorough set of analyses, permission was granted for both of the field trials with ice nucleation-negative bacteria. However, in both instances, although the circumstances were different, local residents who were worried about the release of a GMO in their neighborhoods obtained court orders that temporarily blocked each of the field trials. As a consequence of this delay, both the EPA and the USDA implemented better methodologies for determining the risks
of introducing GMOs into the environment. In a short time, the staffs at these agencies became more proficient at handling and analyzing the data submitted by the applicants. The scientific community, including ecologists, helped the process by initiating research programs that were designed to examine the consequences of the release of organisms into model environments, and scientific organizations formulated frameworks for deciding whether a particular GMO would have an adverse effect on the environment.

Eventually, in 1987, the field trials with ice nucleation-negative bacteria were conducted at sites in California. The results indicated that these GMOs were not dispersed to off-site locations, nor did they persist at the site of application. At one site, the freezing temperature of the test plants was lowered by 1°C. However, for a number of reasons, genetically engineered ice-minus bacteria have not been used to protect crop plants from frost damage.

**FIELD TRIALS IN 1987 - ICE® WORKED & NO DELETERIOUS PROBLEMS**

**BUT.... not without destroyed fields!**

**Security beefed up for genetically engineered crops**

**Last target was ice-minus bacterium**

It has been 15 years since activists targeted genetically engineered crops at a UC campus. In 1987-1988, UC Berkeley researchers working with a Bay Area biotechnology company faced considerable public protest over field-testing of strawberry plants that had been genetically engineered with the "ice minus" bacterium for frost resistance. Strawberry and potato plants carrying the ice-minus bacterium were destroyed in Brentwood and Tule Lake, respectively.
THE OUTCOME 2005!!

FROST CONTINUES TO CAUSE MAJOR LOSSES TO CROPS WORLD-WIDE

ICE- BACTERIA HAS BECOME A HISTORICAL FOOTNOTE—LONG FORGOTTEN & NOT USED. WHY? REGULATORY COSTS, PORTLY BY IT BEING CLASSIFIED AS A PESTICIDE

But... ICE- BACTERIA FOUND A NICHE ON THE SKI SLOPES!

IRONY?
What Exactly Is Snomax And How Does It Work?

Snomax
YORK INTERNATIONAL
Thus, water does not in fact freeze at 0°C, as we tend to think. The water droplet first has to reach its nucleation temperature. Energy is given off as the droplet cools (1 calorie per gram of water). When the crystal forms around the nucleator, surplus energy (80 calories per gram of water) is released, raising the temperature of the droplet to 0°C. 0°C is thus the temperature at which water *stays frozen* (cf. graph on the left).

**Snomax enables water droplets to freeze faster and more completely on coming out of the snow gun**

- 2°C = water temperature
- -3°C = freezing point for water with Snomax
- -8°C = freezing point for water without Snomax

Water molecules in liquid form

Molecules form a hexagonal array
Adding Snomax increases the number of nucleators by 1,000 to 100,000 (test carried out on 73 samples of Snomax-treated and untreated water).

Advantages of Snomax

Using Snomax optimises snow covering. By enhancing droplet crystallisation, Snomax maximises snow production in terms of volume. Numerous tests carried out in ski resorts over the last 15 years have shown an average volume increase of 20%. These tests were performed at constant output on snowmakers of the same design.

With automated YORK equipment, using Snomax enhances programmed snow quality, while producing a better quality of snow on the ground. In this way, output rates can be raised while still keeping an excellent quality of snow.

Snomax also enables weather conditions, be they marginal or cold, to be made the best of. Resorts using Snomax save on water and on both the energy and the time spent on snowmaking.

Adding Snomax to the snowmaking water not only improves and increases the quality and durability of the snow produced, but also makes it less affected by abrasion and by the freeze-thaw cycle.

Moreover, the work of grooming and tilling the snow is improved and run-off from snow piles is reduced. The snow made using Snomax is drier and more homogeneous – as many droplets as possible crystallising – and so of better quality.
Snomax® is a biodegradable product of natural origin, entirely harmless for both people and plants. It has been used in many North American ski resorts for over 15 years.

More than 35 independent research studies, conducted in the U.S.A. and in other countries over a period of more than 8 years, including the Biplink report, the Nakiska study and Walker & Wilkinson's study, have all come to the same conclusion: Snomax entails neither health nor environmental risk. It is without residual impact, and the ecosystem is conserved.

Furthermore, Snomax® is a natural product. It is noteworthy that health and environment regulators in the U.S., Canada, Switzerland, Norway, Sweden, Finland, Australia, New Zealand, Andorra, the Czech Republic and Japan have all subjected Snomax to control investigation and made no objection to its being used.

**Operation and use**

Snomax comes in the form of pellets to be dissolved in water. A first mix is prepared in a 500 litre tank and injected directly into the water pipes, in proportion to the output rate onto the ski-slopes.

The Snomax is injected independently by a Snomax Injection System, comprising a mixing tank, a mixing pump and an injection pump controlled by the installation's flowmeter, to guarantee optimal injection rates.

The injection system may also be run by the YORK software: start/stop, quality changes and alarm (according to the configuration of the existing equipment).

One case of Snomax contains 10 bags, to treat 3,800 m³ of water in all.

**Snomax, a partner on the Olympic sites**

Snomax was the official supplier for snowmaking at the Winter Olympics in Calgary '88, Albertville '92 and Lillehammer '94. At Lillehammer, the snow on the runs for the Alpine events was 98% synthetic, and 100% of this was produced using Snomax. Snomax will also be there at the 2002 Salt Lake City Winter Games.

**SNOMAX**

- IT'S JUST BETTER SNOW!
Mendel is currently licensing WeatherGard™ genes. WeatherGard™ genes are regulatory genes that allow plants to be engineered to be more tolerant to drought, freezing, and high salt soils. Thus, they are "proof of concept" that regulatory genes control valuable agricultural traits.

Crops with WeatherGard™ genes can grow normally under low moisture conditions and resist frost damage. This not only increases crop yields and grain quality, but also expands the available area suitable for sustainable agriculture. WeatherGard™ crops may also reduce the need for irrigation water. The potential value of drought and frost tolerance is huge. As little as a 1% increase in grain production due to better drought and frost tolerance will generate $3 to $4 billion per year.

Drought is one of the most systematic plagues affecting agriculture. It is estimated that global crop losses due to drought exceed $10 billion annually. Between 1978 and 1995, average crop losses due to drought in the US exceed $1.2 billion each year. Since 1995, the US has experienced 3 major droughts with agricultural losses exceeding $1 billion each. The 1999 drought in the eastern US cost farmers as much as $1.2 billion in lost income. The drought of 1998 caused $6-$9 billion in agricultural losses. Finally, the severe drought in the fall of 1995 through the summer of 1996 cost farmers and ranchers approximately $5 billion.

Losses due to frost are more variable, but still significant. Estimates of annual crop losses due to frost damage range from $200 million to $1 billion per year.

Mendel is aggressively working with academic and industrial collaborators to bring WeatherGard™ technology to the world's farmers. We expect WeatherGard™ crops to be important contributors to increased global food production.
Californians are in crisis mode. Is the cause drive-by shootings, tomatoes, or the mother of all quakes? Worse, much worse: Coffee prices are going through the roof. Starbucks has just raised prices again, pushing the cost of their decaf house blend to $10.65 a pound. And that's likely to be just the beginning.

Coffee futures prices in early June surpassed $3 a pound for the first time since mid-1977 in a market increasingly concerned about the potential for summer frosts in Brazil, the world's largest producer. Severe frosts there in 1994 damaged coffee trees, which generally take at least three years to resume good yields.

Oh, well, just an act of God, with no one to blame, right? Wrong.

High technology might have been able to mitigate frost damage, had U.S. regulators at the Environmental Protection Agency not discouraged R&D 15 years ago on an innovative biotechnology product.

In the early 1980's scientists at the University of California and in industry tried a new approach to limiting frost damage. They knew that a harmless bacterium which normally lives on many plants contains an "ice nucleation" protein that promotes frost damage to plants. The scientists sought to produce a variant of the bacterium that lacked the ice-nucleation protein. They reasoned that spraying this variant bacterium (called "ice-minus")) might prevent frost damage by displacing the common ice-promoting kind.

Using very precise biotechnology techniques called recombinant DNA, or "gene splicing," the researchers excised the gene for the ice nucleation protein and planned field tests of the ice-minus bacteria. Government regulations were to pose insurmountable barriers to commercial development, however. The EPA classified as a pesticide the obviously innocuous ice-minus bacteria which were to be tested on small, fenced-off plots of potatoes and strawberries. The EPA reasoned that the naturally-occurring, ubiquitous ice-plus bacterium is a "pest" because its ice-nucleation protein promotes ice crystal formation. Therefore, other bacteria intended to displace it would be a "pesticides." (This is the kind of convoluted reasoning that could lead EPA to regulate outdoor trash cans as a pesticide because litter is an environmental pest.)

At the time, scientists within and outside the EPA were unanimous about the safety of the test. Nonetheless, the field trial was subjected to an extraordinary, lengthy and burdensome review just because the organism was gene-spliced, something that does not apply to bacteria with identical traits but constructed with older, cruder techniques.

And even after the EPA finally granted its approval for testing in the field, the agency conducted elaborate, intrusive and unnecessary monitoring of the field trials.

The ice-minus bacteria were safe and effective at preventing frost damage in field trials. But further research was discouraged by the combination of onerous government regulation, inflated expense of doing the experiments and the prospect of huge downstream costs of pesticide registration.

The product was never commercialized, one reason that the supply—and therefore, the price—of citrus, berries, coffee and other crops remains a hostage to the vagaries of killing frosts.

These effects of government policies should provide food for thought as you sip that increasingly pricey cup of java.

Henry Miller is a senior research fellow at the Hoover Institution and author of "Policy Controversy in Biotechnology: An Insider's View."
GENETIC ENGINEERING
yeasts
# Using Yeast as Factories and "Catalysts"

## Table 36.1: Fungi

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Typical Examples</th>
<th>Key Characteristics</th>
<th>Approximate Number of Living Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascomycota</td>
<td>Yeasts, truffles, molds</td>
<td>Develop by sexual means; ascospores are formed inside a sac called an ascus; asexual reproduction is also common</td>
<td>32,000</td>
</tr>
<tr>
<td>Imperfect fungi</td>
<td>Aspergillus, Penicillium</td>
<td>Sexual reproduction has not been observed; most are thought to be ascomycetes that have lost the ability to reproduce sexually</td>
<td>17,000</td>
</tr>
<tr>
<td>Basidiomycota</td>
<td>Mushrooms, toadstools, ruts</td>
<td>Develop by sexual means; basidiospores are borne on club-shaped structures called basidia; the terminal hyphal cell that produces spores is called a basidium; asexual reproduction occurs occasionally</td>
<td>22,000</td>
</tr>
<tr>
<td>Zygomycota</td>
<td>Rhizopus (black bread mold)</td>
<td>Develop sexually and asexually; male and female hyphae lack septa, except for reproductive structures; fusion of hyphae leads directly to formation of a zygote, in which meiosis occurs just before it germinates</td>
<td>1050 -</td>
</tr>
</tbody>
</table>

## Diagram

```
Sugars

\[ \text{Triose phosphate} \]

\[ \text{Phosphoglycerate} \]

\[ \text{Pyruvate} \]

\[ \text{CO}_2 \]

\[ \text{NAD}^+ \]

\[ \text{NADH + H}^+ \]

\[ \text{O}_2 \text{ Absent} \]

\[ \text{Fermentation} \]

\[ \text{Pyruvate} \]

\[ \text{Acetaldehyde} \]

\[ \text{Ethanol} \]

\[ \text{O}_2 \text{ present} \]

\[ \text{O}_2 \text{ absent} \]

\[ \text{Energy (ATP)} \]
```

- Sugars
- Triose phosphate
- Phosphoglycerate
- Pyruvate
- CO₂
- NAD⁺
- NADH + H⁺
- O₂ Absent
- Fermentation
- Pyruvate
- Acetaldehyde
- Ethanol
- Oxygen present
- Oxygen absent
- Energy (ATP)
VACCINES
- Hepatitis B virus surface antigen
- Malaria circumsporozoite protein
- HIV-1 envelope protein

DIAGNOSTICS
- Hepatitis C virus protein
- HIV-1 antigens

HUMAN THERAPEUTIC AGENTS
- Epidermal growth factor
- Insulin
- Insulin-like growth factor
- Platelet-derived growth factor
- Proinsulin
- Fibroblast growth factor
- Granulocyte-macrophage colony-stimulating factor
- α1 antitrypsin
- Blood coagulation factor XIIIa
- Hirudin
- Human growth factor
- Human serum albumin

Advantages over Bacteria?
Vectors?
Switches?
### Table 6.5 The origins of the different kinds of alcoholic beverages.

<table>
<thead>
<tr>
<th>Alcoholic beverage</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-distilled</strong></td>
<td></td>
</tr>
<tr>
<td>Beer</td>
<td>On germination, starch in barley grains is converted to sugar, which is extracted by boiling in water to produce wort. And this is fermented.</td>
</tr>
<tr>
<td>Cider</td>
<td>Fermentation of apple juice</td>
</tr>
<tr>
<td>Wine</td>
<td>Fermentation of grape juice</td>
</tr>
<tr>
<td>Sake</td>
<td>Starch in steamed rice is hydrolysed with <em>Aspergillus oryzae</em> and the sugars released are fermented with yeast</td>
</tr>
<tr>
<td><strong>Distilled</strong></td>
<td></td>
</tr>
<tr>
<td>Whisky (Scotch)</td>
<td>Distillation of alcohol produced from barley</td>
</tr>
<tr>
<td>Whiskey—Irish</td>
<td>Pot still whiskey produced from alcohol derived from a mixture of barley, wheat and rye. Grain whiskey produced from alcohol derived from maize</td>
</tr>
<tr>
<td>—Rye</td>
<td>Produced from alcohol derived from rye</td>
</tr>
<tr>
<td>—Bourbon</td>
<td>Produced from alcohol derived from maize</td>
</tr>
<tr>
<td>Rum</td>
<td>Distillation of fermented molasses, a by-product of sugar cane refining</td>
</tr>
<tr>
<td>Vodka</td>
<td>Distillation of alcohol produced from any non-grain carbohydrate source, e.g. potatoes</td>
</tr>
<tr>
<td>Gin</td>
<td>Distillation of alcohol derived from maize or rye and redistillation in presence of herbs and juniper berries</td>
</tr>
<tr>
<td>Tequila</td>
<td>Distillation of fermented extracts of Mexican cactus</td>
</tr>
</tbody>
</table>

![Diagram](https://via.placeholder.com/150)

**Figure 13.10** Industrial production of fructose and alcohol from starch.
What happens to pyruvate, the product of glycolysis? In the presence of oxygen, pyruvate is oxidized to acetyl-CoA, which enters the Krebs cycle. In the absence of oxygen, pyruvate is instead reduced, accepting the electrons extracted during glycolysis and carried by NADH. When pyruvate is reduced directly, as in muscle cells, the product is lactate. When CO₂ is first removed from pyruvate and the product, acetaldehyde, is then reduced, as in yeast cells, the product is ethanol.
What is a YAC?

Haven't yet. Why?
GENETIC ENGINEERING
ANIMALS & PLANTS
**Animals vs. Plants** can also be used as factories to produce large amounts of human proteins.

**Molecular Pharming**

1. **B-lactoglobulin promoter**
2. **Sheep ovum**
3. **DNA is injected into pronucleus**
4. **Implant into foster mother**
5. **Transgenic progeny are identified by PCR**
6. **Expression of YFG is restricted to mammary tissue**
7. **Obtain milk from transgenic animals**
8. **YFG product is secreted into milk**
9. **Fractionate milk proteins**
10. **Pure YFG product**

**Reasons**

1. **Proteins need to be modified after translation to be active** - only eukaryotic cells can do this.
2. **Bacteria need big fermentors to elaborate protein purification schemes** - then animals vs. plants can be used for this purpose with special processing/machinery.
3. **Proteins in plants (e.g. soy, seeds) indefinitely stable - can be stored cheaply (in green cheaply) for long periods of time.**
### TABLE 3.1 Potential uses of transgenic animals for pharmaceutical production.

<table>
<thead>
<tr>
<th>Species</th>
<th>Theoretical Yield (g/yr of Raw Protein)</th>
<th>Examples of Products Under Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>250</td>
<td>Monoclonal antibodies, Lysozyme, Growth hormone, Insulin, Human serum albumin</td>
</tr>
<tr>
<td>Rabbit</td>
<td>20</td>
<td>Calcitonin, Superoxide dismutase, Erythropoietin, Growth hormone, IL-2, α-glucosidase</td>
</tr>
<tr>
<td>Goat</td>
<td>4,000</td>
<td>Antithrombin III, Tissue plasminogen activator, Monoclonal antibodies, α-1-Antitrypsin, Growth hormone</td>
</tr>
<tr>
<td>Sheep</td>
<td>2,500</td>
<td>α-1-Antitrypsin</td>
</tr>
<tr>
<td>Cow</td>
<td>80,000</td>
<td>Human serum albumin, Lactoferrin, α-Lactalbumin</td>
</tr>
</tbody>
</table>


And other uses—enhanced milk from transgenic animals!
Table 19.3  Some exogenous proteins that have been expressed in the mammary glands of transgenic animals

- Antithrombin III
- Calcitonin
- Erythropoietin
- Factor IX
- Factor VIII
- Fibrinogen
- Glucagon-like peptide
- Granulocyte colony-stimulating factor
- Growth hormone
- Hemoglobin
- Human serum albumin
- Insulin
- Insulin-like growth factor 1
- Interleukin 2
- Lactoferrin
- Lysozyme
- Monoclonal antibodies
- Nerve growth factor β
- Protein C
- Superoxide dismutase
- Tissue plasminogen activator
- α1-Antitrypsin
- α-Glucosidase
- α-Lactalbumin

Table 19.2  Milk production and estimated recombinant protein yields from organisms used for the expression of transgenes in mammary glands

<table>
<thead>
<tr>
<th>Organism</th>
<th>Annual milk yield (liters)</th>
<th>Estimated recombinant protein per female (kg/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td>Pig</td>
<td>300</td>
<td>1.5</td>
</tr>
<tr>
<td>Sheep</td>
<td>500</td>
<td>2.5</td>
</tr>
<tr>
<td>Goat</td>
<td>900</td>
<td>4</td>
</tr>
<tr>
<td>Cow</td>
<td>10,000</td>
<td>60</td>
</tr>
</tbody>
</table>

Advantages over bacteria?
Producing TPA in a Goat

Advantages:

1. **Cost** → no special equipment needed
2. **Mammalian Gene active in Mammalian Cell** → use goat switch for controls
3. By-product of other uses of goats
4. Eukaryotic Protein Modification Processes

Out → generation time long to establish transgenic farm animals & only few offspring; scale-up hard...but....
Designers milk from transgenic clones

Biotechnology gets a step closer in the pre-harvest production of "new milks" by generating cows that overexpress casein proteins in their milk.

Table 1. Potential modifications of milk composition by gene addition, with expected functional outcome (modified from ref. 2).

<table>
<thead>
<tr>
<th>Modification</th>
<th>Functional consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction of casein genes</td>
<td>Increase in protein and calcium content. Reduction in micelle size, enhancement of heat stability</td>
</tr>
<tr>
<td>Increase ratio of $\alpha$-casein to $\beta$-casein or concomitant increase of all caseins by transferring casein locus</td>
<td></td>
</tr>
<tr>
<td>Modification of casein genes</td>
<td>Increase in calcium content, micelle size, and stability of milk. Enhanced amphiplicity of $\beta$-casein increases its emulsifying and foaming properties</td>
</tr>
<tr>
<td>Add phosphorylation sites</td>
<td></td>
</tr>
<tr>
<td>Introduction of protease (chymosin) cleavage sites</td>
<td>Increase in rate of cheese-ripening</td>
</tr>
<tr>
<td>Deletion of protease (plasmin) site from $\beta$-casein</td>
<td>Increase in emulsifying properties. Elimination of bitter flavor in cheese</td>
</tr>
<tr>
<td>Introduction of other functional proteins</td>
<td>Milk with antimicrobial activity</td>
</tr>
<tr>
<td>Add lysozyme, lactoferrin, or lysozymeprotein</td>
<td></td>
</tr>
<tr>
<td>Add reversibly inactive lactase that is activated in gastrointestinal tract upon ingestion of milk</td>
<td>Elimination of sweet taste of lactose-hydrolyzed milk and alleviation of lactose intolerance symptoms</td>
</tr>
</tbody>
</table>

Table 19.1 Protein composition (grams/liter) of milk from cattle and sheep

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Cattle</th>
<th>Sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_{S1}$-Casein</td>
<td>10.0</td>
<td>12.0</td>
</tr>
<tr>
<td>$\alpha_{S2}$-Casein</td>
<td>3.4</td>
<td>3.8</td>
</tr>
<tr>
<td>$\kappa$-Casein</td>
<td>3.9</td>
<td>4.6</td>
</tr>
<tr>
<td>$\beta$-Casein</td>
<td>10.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Major whey proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha$-Lactalbumin</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>$\beta$-Lactalbumin</td>
<td>3.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Other proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum albumin</td>
<td>0.4</td>
<td>Unknown</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Trace</td>
<td>Unknown</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>0.1</td>
<td>Unknown</td>
</tr>
<tr>
<td>Immunglobulins</td>
<td>0.7</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
Myogenic expression of an injectable protease-resistant growth hormone–releasing hormone augments long-term growth in pigs

Ruxandra Dragha-Akili, Marta L. Fiorotto, Leigh Anne Hill, P. Brandon Malone, Daniel R. Deaver, and Robert J. Schwartz
Production of transgenic animals by injecting eggs with genes is not efficient.

Table 11.3 Efficiency of production of transgenic animals by microinjection of a growth hormone gene. (Adapted from Hammer et al. 1985.)

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. of ova injected</th>
<th>No. of offspring</th>
<th>No. of transgenic offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>1907</td>
<td>218</td>
<td>28</td>
</tr>
<tr>
<td>Sheep</td>
<td>1032</td>
<td>73</td>
<td>1</td>
</tr>
<tr>
<td>Pig</td>
<td>2035</td>
<td>192</td>
<td>20</td>
</tr>
</tbody>
</table>

Overall efficiency (%)

Key
- Cattle
- Figs
- Sheep
- Mice

Limit use of molecular pharming for pharmaceutical production.
16.4 A Clone and Her Offspring

Although Dolly herself (right) is a clone with only one parent, she has mated and given birth to "normal" offspring (the lamb on the left), proving the genetic viability of cloned mammals.

**Conclusion:** Differentiated animal cells are totipotent in nuclear transplant experiments.
**Using Cloning & Recombinant DNA to Make Transgenic Pharm Animals**

1. **Expression vector** has the gene for resistance to the antibiotic, neomycin.

2. Add expression vector to sheep somatic cells.

3. Add neomycin, which kills all cells that do not have the vector.

4. Cells resistant to being killed contain the vector and are transgenic; they grow and divide.

5. A transgenic cell is used as nuclear source for fusion with an enucleated egg.

6. An embryo develops...

7. ...and is implanted in surrogate female.

8. Clone produces abundant α-1 AT in milk.

**17.15 Production of Transgenic Clones for "Pharming"**
The production of transgenic animals involves a combination of DNA technology and reproductive technology.
TRANSPLANTS OF TISSUES from animals to humans (xenotransplants) have been attempted experimentally using a variety of donor animals, from frogs to baboons and pigs. Most efforts quickly failed. But doctors may soon perfect ways to transplant organs, such as the heart, from specially bred pigs.
Knocking out xenograft rejection

Two reports on the knockout of one allele of the α1,3-galactosyltransferase gene in pigs bring us one step closer to the transplantation of pig organs into people.

Figure 1. α1,3Gal in the fate of xenografts and the mechanisms of tissue injury. (A) Fate of xenografts. The impact of immune response on xenografts depends on the type of graft. Organ xenografts are subject to vascular rejection of various types thought to be induced by anti-donor antibodies and cellular rejection caused by T cells. Cell and tissue xenografts are subject to primary non-function, thought to be caused by macrophages and cellular rejection. Expression of α1,3Gal and the action of anti-Gal antibodies is expected to have a far more profound impact on the fate of organ grafts than on the fate of cell or tissue grafts. (B) The role of α1,3Gal in hyperacute and acute vascular rejection. Hyperacute rejection is caused by binding of large amounts of antibody, consisting predominantly of anti-α1,3Gal, to graft blood vessels, activating large amounts of complement. It is prevented by anything that inhibits antibodies or complement. Acute vascular rejection is caused by binding of antibodies to the graft with or without complement. The antibodies causing acute vascular rejection may be directed against α1,3Gal or against other xenogenic proteins. Acute vascular rejection is not prevented by complement inhibitors, but may be inhibited by depleting antibodies or by modifying antigenic targets, as might be seen in the α1,3GT-knockout pig.

z.e. nature.com  •  MARCH 2002  •  VOLUME 20  •  nature biotechnology  •  231
Targeted disruption of the \( \alpha_{1,3} \)-galactosyltransferase gene in cloned pigs

Yifan Dai\(^{1*} \), Todd D. Vaught\(^{1} \), Jeremy Boone\(^{1} \), Shu-Hung Chen\(^{1} \), Carol J. Phelps\(^{1} \), Suyapa Ball\(^{1} \), Jeff A. Monahan\(^{1} \), Peter M. Jobst\(^{1} \), Kenneth J. McCrea\(^{2} \), Ashley E. Lamborn\(^{1} \), Jamie L. Cowell-Lucero\(^{1} \), Kevin D. Wells\(^{1} \), Alan Colman\(^{2} \), Irina A. Polejaeva\(^{1} \), and David L. Ayares\(^{1} \)

Galactose-\( \alpha_{1,3} \)-galactose (\( \alpha_{1,3} \)Gal) is the major xenotransplantation. Disruption of the gene encoding pig \( \alpha_{1,3} \)-galactosyltransferase (\( \alpha_{1,3} \)GT) by homologous recombination is a means to completely remove the \( \alpha_{1,3} \)Gal epitopes from xenografts. Here we report the disruption of one allele of the pig \( \alpha_{1,3} \)GT gene in both male and female porcine primary fetal fibroblasts. Targeting was confirmed in 17 colonies by Southern blot analysis, and 7 of them were used for nuclear transfer. Using cells from one colony, we produced six cloned female piglets, of which five were of normal weight and apparently healthy. Southern blot analysis confirmed that these five piglets contain one disrupted pig \( \alpha_{1,3} \)GT allele.

Figure 3. Five \( \alpha_{1,3} \)GT gene knockout piglets at 2 weeks of age.
Table 1. Literature survey of developmental problems in cloned animals

<table>
<thead>
<tr>
<th>Species</th>
<th>Percentage healthy animals (healthy/total born)</th>
<th>Problems (% of reported problem cases after birth)</th>
<th>Follow-up period</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>100/10</td>
<td>None</td>
<td>None</td>
<td>4 weeks</td>
</tr>
<tr>
<td>Cattle</td>
<td>100/20</td>
<td>None</td>
<td>Diabetes (100). This animal survived into adulthood</td>
<td>2 months</td>
</tr>
<tr>
<td>Cattle</td>
<td>100/5</td>
<td>None</td>
<td>Pulmonary hypertension, dilated cardiomyopathy (17)</td>
<td>7 months</td>
</tr>
<tr>
<td>Cattle</td>
<td>100/5</td>
<td>None</td>
<td>Internal hemorrhage umbilical artery (108)</td>
<td>8 months</td>
</tr>
<tr>
<td>Cattle</td>
<td>100/5</td>
<td>None</td>
<td>Viral infection (50), dystocia (60)</td>
<td>8-15 months</td>
</tr>
<tr>
<td>Cattle</td>
<td>54/134/5</td>
<td>None</td>
<td>Dystocia (15), bacterial infection (6), kidney problems (42)</td>
<td>1-4 years</td>
</tr>
<tr>
<td>Cattle</td>
<td>50/1</td>
<td>None</td>
<td>Overzealized, leg malformation (100)</td>
<td>NA</td>
</tr>
<tr>
<td>Cattle</td>
<td>50/4</td>
<td>None</td>
<td>Pneumonia (25), drawing in amniotic fluid (50), dystocia (25)</td>
<td>10-12 months</td>
</tr>
<tr>
<td>Cattle</td>
<td>44/11</td>
<td>None</td>
<td>Heart defects (57), liver fibrosis (29), pneumonia (7), osteoporosis (21), joint defects (14), anemia (42)</td>
<td>2-12 months</td>
</tr>
<tr>
<td>Cattle</td>
<td>40/4</td>
<td>None described</td>
<td>Viral infection (68)</td>
<td>2-4 months</td>
</tr>
<tr>
<td>Sheep</td>
<td>100/1</td>
<td>None</td>
<td>Thymic atrophy, lymphoid hypoplasia (100)</td>
<td>4 weeks</td>
</tr>
<tr>
<td>Sheep</td>
<td>100/1</td>
<td>None</td>
<td>Viral infection (68)</td>
<td>1 year</td>
</tr>
<tr>
<td>Sheep</td>
<td>0/0</td>
<td>None described</td>
<td>NA</td>
<td>1 month</td>
</tr>
<tr>
<td>Sheep</td>
<td>0/0</td>
<td>None described</td>
<td>NA</td>
<td>1 year</td>
</tr>
<tr>
<td>Goats</td>
<td>100/1</td>
<td>None</td>
<td>Kidney, liver, and brain defects</td>
<td>6 years</td>
</tr>
<tr>
<td>Goats</td>
<td>100/1</td>
<td>None</td>
<td>Kidney and liver defects</td>
<td>3 weeks</td>
</tr>
<tr>
<td>Goats</td>
<td>100/1</td>
<td>None</td>
<td>Kidney and liver defects</td>
<td>3 years</td>
</tr>
<tr>
<td>Goats</td>
<td>100/1</td>
<td>None</td>
<td>Kidney and liver defects</td>
<td>3 years</td>
</tr>
<tr>
<td>Goats</td>
<td>100/1</td>
<td>None</td>
<td>Kidney and liver defects</td>
<td>6 months</td>
</tr>
<tr>
<td>Goats</td>
<td>100/1</td>
<td>None</td>
<td>Kidney and liver defects</td>
<td>6 months</td>
</tr>
<tr>
<td>Goats</td>
<td>100/1</td>
<td>None</td>
<td>Kidney and liver defects</td>
<td>6 months</td>
</tr>
<tr>
<td>Goats</td>
<td>100/1</td>
<td>None</td>
<td>Kidney and liver defects</td>
<td>6 months</td>
</tr>
<tr>
<td>Pigs</td>
<td>100/1</td>
<td>None</td>
<td>Bacterial infection in the lungs (100)</td>
<td>3 years</td>
</tr>
<tr>
<td>Pigs</td>
<td>100/1</td>
<td>None</td>
<td>Bacterial infection in the lungs (100)</td>
<td>1 year</td>
</tr>
<tr>
<td>Pigs</td>
<td>100/1</td>
<td>None</td>
<td>Bacterial infection in the lungs (100)</td>
<td>1 year</td>
</tr>
<tr>
<td>Pigs</td>
<td>100/1</td>
<td>None</td>
<td>Bacterial infection in the lungs (100)</td>
<td>1 year</td>
</tr>
<tr>
<td>Pigs</td>
<td>100/1</td>
<td>None</td>
<td>Bacterial infection in the lungs (100)</td>
<td>1 year</td>
</tr>
<tr>
<td>Pigs</td>
<td>100/1</td>
<td>None</td>
<td>Bacterial infection in the lungs (100)</td>
<td>1 year</td>
</tr>
<tr>
<td>Mice</td>
<td>100/8</td>
<td>None</td>
<td>Obesity (100). This was not a lethal disorder</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Mice</td>
<td>100/4</td>
<td>None</td>
<td>Enlarged placenta (20)</td>
<td>6 months</td>
</tr>
<tr>
<td>Mice</td>
<td>100/4</td>
<td>None</td>
<td>Enlarged placenta (20)</td>
<td>6 months</td>
</tr>
<tr>
<td>Mice</td>
<td>100/4</td>
<td>None</td>
<td>Enlarged placenta (20)</td>
<td>2 months</td>
</tr>
<tr>
<td>Mice</td>
<td>100/4</td>
<td>None</td>
<td>Enlarged placenta (20)</td>
<td>2 months</td>
</tr>
<tr>
<td>Mice</td>
<td>100/4</td>
<td>None</td>
<td>Enlarged placenta (20)</td>
<td>2 months</td>
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<td>100/4</td>
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<td>2 months</td>
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<tr>
<td>Mice</td>
<td>100/4</td>
<td>None</td>
<td>Enlarged placenta (20)</td>
<td>2 months</td>
</tr>
<tr>
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<td>100/4</td>
<td>None</td>
<td>Enlarged placenta (20)</td>
<td>2 months</td>
</tr>
<tr>
<td>Mice</td>
<td>100/4</td>
<td>None</td>
<td>Enlarged placenta (20)</td>
<td>2 months</td>
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<tr>
<td>Total</td>
<td>77/259</td>
<td>None</td>
<td>Respiratory failure/umbilical hernia (40), failure to foster (20)</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Total</td>
<td>77/259</td>
<td>None</td>
<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Total</td>
<td>77/259</td>
<td>None</td>
<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Total</td>
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<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
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<td>77/259</td>
<td>None</td>
<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Total</td>
<td>77/259</td>
<td>None</td>
<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Total</td>
<td>77/259</td>
<td>None</td>
<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Total</td>
<td>77/259</td>
<td>None</td>
<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Total</td>
<td>77/259</td>
<td>None</td>
<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
</tr>
<tr>
<td>Total</td>
<td>77/259</td>
<td>None</td>
<td>Respiratory failure (100)</td>
<td>&gt;3 months</td>
</tr>
</tbody>
</table>
TABLE 2.1 State of the art of transgenic technology for selected organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Transfection</th>
<th>Viral vectors</th>
<th>Transposon</th>
<th>ES cells</th>
<th>Nuclear transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>4*</td>
<td>2</td>
<td>1</td>
<td>4*</td>
<td>2</td>
</tr>
<tr>
<td>Cow</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Sheep</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Goat</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Pig</td>
<td>3</td>
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<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>Chicken</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Atlantic salmon</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Channel catfish</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tilapia</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>Zebrafish</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Crustaceans</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Mollusks</td>
<td>1</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Drosophila</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mosquito</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE:  
0: No significant progress.  
1: Has been accomplished experimentally (proof of concept).  
2: Routine experimental use.  
3: Commercialization sought.  
4: Widespread production.  
*For experimental uses.  
See (Dove, 2000)
<table>
<thead>
<tr>
<th>Species</th>
<th>Foreign Gene</th>
<th>Desired Effect and Comments</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic salmon</td>
<td>AFP, AFP salmon GH</td>
<td>Cold tolerance, increased growth and feed efficiency</td>
<td>United States, Canada</td>
</tr>
<tr>
<td>Coho salmon</td>
<td>Chinook salmon GH + AFP</td>
<td>After 1 year, 10- to 30-fold growth increase</td>
<td>Canada</td>
</tr>
<tr>
<td>Chinook salmon</td>
<td>AFP salmon GH</td>
<td>Increased growth and feed efficiency</td>
<td>New Zealand</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>AFP salmon GH</td>
<td>Increased growth and feed efficiency</td>
<td>United States, Canada</td>
</tr>
<tr>
<td>Cutthroat trout</td>
<td>Chinook salmon GH + AFP</td>
<td>Increased growth</td>
<td>Canada</td>
</tr>
<tr>
<td>Tilapia</td>
<td>AFP salmon GH</td>
<td>Increased growth and feed efficiency, stable inheritance, increased growth and stable inheritance</td>
<td>Canada, United Kingdom</td>
</tr>
<tr>
<td>Salmon</td>
<td>Rainbow trout lysosome gene and flounder pipecaud gene</td>
<td>Disease resistance, still in development</td>
<td>United States, Canada</td>
</tr>
<tr>
<td>Striped bass</td>
<td>Insect genes</td>
<td>Disease resistance, still in early stages of research</td>
<td>United States</td>
</tr>
<tr>
<td>Channel catfish</td>
<td>GH</td>
<td>33% growth improvement in culture conditions</td>
<td>United States</td>
</tr>
<tr>
<td>Common carp</td>
<td>Salmon and human GH</td>
<td>150% growth improvement in culture conditions; improved disease resistance; tolerance of low oxygen levels</td>
<td>China, United States</td>
</tr>
<tr>
<td>Goldfish</td>
<td>GH, AFP</td>
<td>Increased growth</td>
<td>China</td>
</tr>
<tr>
<td>Abalone</td>
<td>Coho salmon GH + various promoters</td>
<td>Increased growth</td>
<td>United States</td>
</tr>
<tr>
<td>Oysters</td>
<td>Coho salmon GH + various promoters</td>
<td>Increased growth</td>
<td>United States</td>
</tr>
<tr>
<td>Fish genes used in other life forms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Salmon calcitonin-producing gene</td>
<td>Calcitonin production to control calcium loss from bones</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>Strawberry and potatoes</td>
<td>AFP</td>
<td>Increased cold tolerance</td>
<td>United Kingdom, Canada</td>
</tr>
</tbody>
</table>

Note: The development of transgenic organisms requires the insertion of the gene of interest and a promoter, which is the switch that controls expression of the gene.

Figure 8.11  Comparison of 1-month-old coho salmon siblings: nonengineered fish are at left, transgenic fish are at right. The largest fish (top right) is 41.8 cm in length.

What are the issues with these fish?
Transgenic Fish: A Boon or Threat?

ERIK STOKSTAD'S ARTICLE "ENGINEERED FISH: friend or foe of the environment?" (News Focus, 13 Sept., p. 1797) entertains the premise that the culture of transgenic fish, which grow two to six times faster than conventional fish, "might alleviate pressure on wild stocks." Two key points not addressed by Stokstad challenge this premise.

First, the culture of carnivorous species, such as salmon and trout, already represents a net drain on wild fish populations. Over 2 kg of wild fish are required to produce 1 kg of aquacultured conventional carnivorous fish (1). In North America and Europe, fish are usually reared in high densities and therefore rely completely on manufactured feeds for sustenance. Manufactured feeds for carnivorous species are typically composed of 35% to 50% fish meal and up to 20% fish oil (2). The accelerated growth rate of transgenic fish will necessitate an enormous increase in the volume of feeds and their constituent marine feedstuffs. Fish meal and fish oil are typically made from menhaden and anchovies harvested from the wild. As these species are already being exploited near their maximum sustainable levels (2), using more of them to create even more feed for transgenic fish can hardly be considered an easing of pressure.

Second, on the basis of the Law of Conservation of Matter, increased feed inputs will result in more outputs of waste in aquaculture effluents [e.g., (3)]. Reclamation of aquaculture waste is already problematic. In net-pen culture, for example, untreated wastes are expelled directly into the surrounding waters and commonly cause local eutrophication, buildup in sediments of feed-borne antibiotics, and benthic anoxia (4). Although the degree of these impacts depends on husbandry practices and the hydrodynamics of the site, the potential for serious environmental damage will increase with the increased feed usage required by transgenic fish culture. Add the potential effects of interbreeding between transgenic escapes and wild fish discussed by Stokstad, and transgenic fish culture appears more threat than boon to the wild fisheries.

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References

Dealing with the Risks of Transgenic Fish

ERIK STOKSTAD'S ARTICLE "ENGINEERED FISH: friend or foe of the environment?" (News Focus, 13 Sept., p. 1797) correctly points out the risk to the environment associated with potential releases of genetically modified aquatic animals. This risk is a function of the specific genes, specific species and strain, and environment, and is independent of whether the genes came from genetic engineering, conventional breeding, or inadvertent selection.

The scientific research community must remain attentive to the details of how these very complex problems are being addressed. Researchers can become "collateral damage" to groups with agendas ranging from real environmental concern, to antitechnology, anti-genetically modified organism activists, to crass commercial interests.

In California, State Senator Byron Sher introduced legislation (1) SB-1525 that would have made it "unlawful to import, transport, possess... any live transgenic fish." When it was clear that this legislation would shut down many zebra fish researchers in California, it was amended to allow researchers to get a permit for non-commercial purposes only. This could still affect researchers by impacting zebra fish suppliers like Scientific Hatcheries and Exelixis, along with the added burden of another layer of permits. This bill with its amended variations and reincarnations posed a real risk to scientific research in California, before it was finally stopped for this year.

The proponents of a ban on transgenic fish (2) submitted a petition to the California Fish and Game Commission to adopt a moratorium on "transgenic" fish and stated that the moratorium would "specifically apply... to ornamental aquatic species, such as transgenic zebra fish." Senator Sher's letter of support (3) specified plans for "mass producing a transgenic form of these zebra fish... as "wrong." When the zebra fish research community heard about these plans and showed up at the Fish and Game Commission meeting on 29 August 2002, the proposal was defeated. Efforts are under way to find a solution to the real problem of unwanted gene movement in the environment, without impacting scientific research and other insignificant environmental risk situations.

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References and Notes
2. Letter to R. Trenton, California Fish and Game Commission from the Natural Resources Defense Council (NRDC), Institute for Fishery Resources, Pacific Coast Federation of Fishermen's Associations (PCFFA) and The Ocean Conservancy, 23 July 2002.
Building the Better Bug

Inserting new genes into a few specific insect species could stop some infectious diseases, benefit agriculture and produce innovative materials

by David A. O'Brochta and Peter W. Atkinson

TRANSGENIC INSECTS can be given new characteristics, as illustrated by these five Anobja aegypti mosquitoes. Normal individuals have what appear to be black eyes, the result of large amounts of red pigment. A mutant version of Ae. aegypti has white eyes because of the lack of an enzyme, kynurenine hydroxylase, required to synthesize the red pigment. This white-eyed condition can be altered via the insertion of the gene for the enzyme. The resultant mosquitoes produce enough pigment to have visibly pink eyes. Such eye-color changes merely point out the potential of transgenic technology for producing a strain incapable of transmitting yellow fever or dengue.
STERILE INSECT TECHNIQUE (SIT) can be an effective weapon against pests. Wave after wave of sterile insects, mostly males when possible, far outnumber the fertile members of the same species, and cause most matings to be fruitless. Within a few generations, the pest population is decimated. Traditional breeding programs have made for successful SIT interventions, but transgenic technology has the potential to streamline these procedures.

MAKING TRANSGENIC INSECTS requires the insertion of a gene (blue), carried by a transposable element such as Hermes (red), into a fertilized egg (1). The new genetic material is strategically placed at the polar plasm (2), that section of the egg destined to become the newly nascent insect's own egg cells when it reaches maturity. After numerous divisions of the egg's nuclear material (3), most of it segregates to the periphery, where it will become the nuclei of the cells of the insect's body; two nuclei, however, will migrate to the pole to become the insect's egg cells (4) when it reaches maturity (5). Should these cells have incorporated the transgene, progeny will be transgenic (6).

TRANSGENIC MEDFLY has its natural eye color restored. White-eyed mutants produce red pigment but cannot transport the pigment to the eyes. The red-eyed Medfly on the left is a transgenic that has been given the transposable element piggoBac, which is carrying a normal copy of the gene enabling pigment transport to the eye.
Sterile Mosquitoes

Mosquitoes that cannot harbor malaria pathogens

ISSUES?
<table>
<thead>
<tr>
<th>Animal</th>
<th>Number of Citations</th>
<th>Ability to Become Feral</th>
<th>Likelihood of Escape Captivity</th>
<th>Mobility</th>
<th>Community Disruptions Reported</th>
<th>Level of Concern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insects</td>
<td>1804</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Many</td>
<td>High</td>
</tr>
<tr>
<td>Fish</td>
<td>186</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Many</td>
<td>High</td>
</tr>
<tr>
<td>Mice/Rats</td>
<td>53</td>
<td>High</td>
<td>High</td>
<td>High</td>
<td>Many</td>
<td>High</td>
</tr>
<tr>
<td>Cat</td>
<td>160</td>
<td>High</td>
<td>High</td>
<td>Moderate</td>
<td>Many</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>155</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
<td>Many</td>
<td></td>
</tr>
<tr>
<td>Goat</td>
<td>88</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Some</td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>93</td>
<td>High</td>
<td>Moderate</td>
<td>High</td>
<td>Few</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>8</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Few</td>
<td></td>
</tr>
<tr>
<td>Mink</td>
<td>16</td>
<td>High</td>
<td>High</td>
<td>Moderate</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>11</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Few</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>11</td>
<td>Low</td>
<td>Moderate</td>
<td>Moderate</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>27</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Few</td>
<td>Low</td>
</tr>
<tr>
<td>Cattle</td>
<td>16</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

1 Number of scientific papers dealing with feral animals of this species.
2 Based on number of feral populations reported.
3 Based on ability of organism to evade confinement measures by flying, digging, swimming, or jumping ability for any of the life stages.
4 Relative dispersal distance by walking, running, flying, swimming, or hitchhiking in trucks, trains, boats, etc.
5 Based on worldwide citations reporting community damage and extent of damage.
6 A ranking based on the four contributing factors.
7 Limited to gypsy moth and Africanized honeybee.

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**Potential Risks of Transgenic Animals?**

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Caused the Food Issues as well - Pharma Products?
Figure 8-72 A procedure used to make a transgenic plant. (A) Outlining the process. A disc is cut out of a leaf and incubated in culture with Agrobacteia that carry a recombinant plasmid with both a selectable marker and a desired transgene. The wounded cells at the edge of the disc release substances that attract the Agrobacteria and cause them to inject DNA into these cells. Only those plant cells that take up the appropriate DNA and express the selectable marker gene survive to proliferate and form a callus. The manipulation of growth factors supplied to the callus induces it to form shoots that subsequently root and grow into adult plants carrying the transgene. (B) The preparation of the recombinant plasmid and its transfer to plant cells. An Agrobacterium plasmid that normally carries the T-DNA sequence is modified by substituting a selectable marker (such as the kanamycin-resistance gene) and a desired transgene between the 25-nucleotide-pair T-DNA repeats. When the Agrobacterium recognizes a plant cell, it efficiently passes a DNA strand that carries these sequences into the plant cell using the special machinery that normally transfers the plasmid's T-DNA sequence.
**Pharming in Plants**

Nicotiana benthamiana, a tobacco plant, serves as a biofactory for producing antibodies against cancer.

Table 14.5 A selection of pharmaceutical recombinant human proteins expressed in plant systems.

<table>
<thead>
<tr>
<th>Species</th>
<th>Recombinant human product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco, sunflower (plants)</td>
<td>Growth hormone</td>
<td>Barta et al. 1985</td>
</tr>
<tr>
<td>Tobacco, potato (plants)</td>
<td>Serum albumin</td>
<td>Sijmons et al. 1990</td>
</tr>
<tr>
<td>Tobacco (plants)</td>
<td>Epidermal growth factor</td>
<td>Higo et al. 1993</td>
</tr>
<tr>
<td>Rice (plants)</td>
<td>c-Interferon</td>
<td>Zhu et al. 1994</td>
</tr>
<tr>
<td>Tobacco (cell culture)</td>
<td>Erythropoietin</td>
<td>Matsumoto et al. 1995</td>
</tr>
<tr>
<td>Tobacco (plants)</td>
<td>Haemoglobin</td>
<td>Dieryck et al. 1997</td>
</tr>
<tr>
<td>Tobacco (cell culture)</td>
<td>Interleukins-2 and 4</td>
<td>Magnuson et al. 1998</td>
</tr>
<tr>
<td>Tobacco (root culture)</td>
<td>Placental alkaline phosphatase</td>
<td>Borisjuk et al. 1999</td>
</tr>
<tr>
<td>Rice (cell culture)</td>
<td>α- Antitrypsin</td>
<td>Terashima et al. 1999</td>
</tr>
<tr>
<td>Tobacco (seeds)</td>
<td>Growth hormone</td>
<td>Leite et al. 2000</td>
</tr>
<tr>
<td>Tobacco (chloroplasts)</td>
<td>Growth hormone</td>
<td>Staub et al. 2000</td>
</tr>
</tbody>
</table>

Table 14.7 A selection of recombinant vaccines against animal viruses produced in plants.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host-plant system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpes virus B surface antigen</td>
<td>Tobacco</td>
<td>Mason et al. 1992</td>
</tr>
<tr>
<td>Rabies glycoprotein</td>
<td>Tomato</td>
<td>McGarvey et al. 1995</td>
</tr>
<tr>
<td>Norwalk virus coat protein</td>
<td>Tobacco, potato</td>
<td>Mason et al. 1996</td>
</tr>
<tr>
<td>Foot-and-mouth virus VP1</td>
<td>Arabidopsis</td>
<td>Carrillo et al. 1998</td>
</tr>
<tr>
<td>Cholera toxin B subunit</td>
<td>Potato</td>
<td>Arakawa et al. 1998</td>
</tr>
<tr>
<td>Human cytomegalovirus glycoprotein B</td>
<td>Tobacco</td>
<td>Tackaberry et al. 1999</td>
</tr>
</tbody>
</table>
Figure 19.11 Structures of biologically active alkaloids and the plants that produce them. Source: Kuchan, T. M. 1995. Alkaloid biosynthesis—The basis for metabolic engineering of medicinal plants. Plant Cell 7:1059–1070.
### Table 19.4 Some specialty uses of plant fatty acids and oils

<table>
<thead>
<tr>
<th>Lipid Type</th>
<th>Example</th>
<th>Major and Alternative Sources</th>
<th>Major Uses</th>
<th>Approx. U.S. Market Size (10^3 t)</th>
<th>10^6 US Dollars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium chain (C8-C14)</td>
<td>Lauric acid</td>
<td>Palm kernel, coconut, Cuphea</td>
<td>Detergents</td>
<td>640</td>
<td>320</td>
</tr>
<tr>
<td>Long chain (C22)</td>
<td>Erucic acid</td>
<td>Rapeseed, Crambe</td>
<td>Lubricants, nylon, plasticizers</td>
<td>30</td>
<td>80</td>
</tr>
<tr>
<td>Epoxy</td>
<td>Vernolic acid</td>
<td>Epoxidized soybean oil, Vernonia</td>
<td>Plasticizers</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Hydroxy</td>
<td>Ricinoleic acid</td>
<td>Castor bean, Lesquerella</td>
<td>Lubricants, coatings</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>Trienoic</td>
<td>Linolenic acid</td>
<td>Flax</td>
<td>Coatings, drying agents</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>Low melting solid</td>
<td>Cocoa butter</td>
<td>Cocoa bean, illipe (Shovea stenoptera)</td>
<td>Chocolate, cosmetics</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>Wax ester</td>
<td>Jojoba oil</td>
<td>Jojoba</td>
<td>Lubricants, cosmetics</td>
<td>0.35</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 19.9** Genetic engineering of canola oil that is high in lauric acid, a fatty acid with 12 carbon atoms. By introducing a single gene from the California bay tree, the canola oil was changed from containing 60% oleic acid to 60% lauric acid. This new canola oil resembles the oil found in coconut and oil palm.

Source: Courtesy of T. Voeller, Calgene/Monsanto.
Figure 19.1 Can plants replace plants? In green plants the inputs are carbon dioxide and solar energy, in chemical plants the input is petroleum.

Figure 19.2 Change in the primary sources of industrial chemicals in the United States between 1930 and 1960. Note the rise of oil and the disappearance of plants and decreased importance of coal over this 30-year period. As of 2000, petroleum provides over 95% of organic chemicals used in the United States.
Figure 9.7 Phytoremediation Plants can be a valuable addition to many bioremediation strategies. Some plants degrade environmental pollutants directly, while others simply absorb pollutants and must be removed and disposed of.
Phytodetoxification of hazardous organomercurials by genetically engineered plants

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Received 13 July 1999; accepted 12 November 1999

Methylmercury is a highly toxic, organic derivative found in mercury-polluted wetlands and coastal sediments worldwide. Though commonly present at low concentrations in the substrate, methylmercury can biomagnify to concentrations that poison predatory animals and humans. In the interest of developing an in situ detoxification strategy, a model plant system was transformed with bacterial genes (merA for mercuric reductase and merB for organomercurial lyase) for an organic mercury detoxification pathway. Arabidopsis thaliana plants expressing both genes grow on 50-fold higher methylmercury concentrations than wild-type plants and up to 10-fold higher concentrations than plants that express merB alone. An in vitro assay demonstrated that both transgenes are required for plants to detoxify organic mercury by converting it to volatile and much less toxic elemental mercury.

Bacteria isolated from organic mercury-contaminated environments possess two enzymes that convert methylmercury and other organomercurials to elemental mercury. [Hg(0)] (ref. 19). Elemental mercury is much less toxic than either Hg(II) or organic mercury and rapidly diffuses out of bacterial cells as a result of its volatility. The bacterial mercury-processing enzymes, organomercurial lyase (MerB) and mercuric reductase (MerA), catalyze the following reaction:

\[ \text{MerB} : \text{Lyase} \]
\[ \text{MerA} : \text{Reductase} \]

\[ \text{R-CH}_2\text{Hg}^+ + \text{H}^+ \rightarrow \text{R-CH}_2\text{H}_2 + \text{Hg}^{(II)} \]

\[ \text{Hg}^{(II)} + \text{NADPH} \rightarrow \text{Hg}^{(0)} + \text{NADP}^+ + \text{H}^+ \]

In theory, plants engineered with both genes should extract organomercurials from substrates and transpire Hg(0) into the atmosphere using the same mechanism as bacteria (Fig. 1). Because the atmospheric residence time of Hg(0) is about two years, it can be diluted to trace concentrations before reabsorbing into the terrestrial substrate. Furthermore, the quantity of mercury released from polluted sites can be regulated and well, in all likelihood, be small in comparison with the atmospheric mercury load (~4 x 10^6 kg) (ref. 20).
Figure 17.7 Hand hoeing of weeds. Hand hoeing is backbreaking and time consuming but is still the primary means of weed control in developing countries. This couple in the Luang Prabang province of Laos is weeding upland rice. Note the numerous weeds among the young rice plants. If not removed at this stage the yield will be lost. Source: Courtesy of Eugene Hettel, International Rice Research Institute.

<table>
<thead>
<tr>
<th>Crop</th>
<th>% Crop Losses</th>
<th>1988-1990</th>
<th>Change in Loss*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>3.9</td>
<td>8.8</td>
<td>+4.9</td>
</tr>
<tr>
<td>Maize</td>
<td>13.0</td>
<td>14.5</td>
<td>+1.5</td>
</tr>
<tr>
<td>Cotton</td>
<td>15.5</td>
<td>14.4</td>
<td>-0.6</td>
</tr>
<tr>
<td>Potatoes</td>
<td>5.9</td>
<td>16.1</td>
<td>+10.2</td>
</tr>
<tr>
<td>Rice</td>
<td>27.5</td>
<td>20.7</td>
<td>-6.8</td>
</tr>
<tr>
<td>Soybeans</td>
<td>4.4</td>
<td>10.4</td>
<td>+6.0</td>
</tr>
<tr>
<td>Wheat</td>
<td>5.1</td>
<td>9.3</td>
<td>+4.2</td>
</tr>
<tr>
<td>Average</td>
<td>10.8</td>
<td>13.6</td>
<td>+2.8</td>
</tr>
</tbody>
</table>


Figure 15.1 Most microbes attack only a specific part of the plant and produce characteristic disease symptoms. Tomato, shown here, can be attacked by more than 100 different pathogenic microorganisms. Source: E. B. Buchanan, W. Gruissem, and R. L. Jones, eds. (2000), Plant Biochemistry and Molecular Biology (Rockville, MD: American Society of Plant Physiologists). p. 1104.
ON THE WAY

1. Non-Allergenic Soybeans & Peanuts
2. Caffeineless Coffee
3. Vitamin-Fortified Foods
4. Non-Ripening Fruits (Slower Ripening)
5. Drought-Resistant Crops
6. Cold-Resistant Crops
7. Fungal-Resistant Crops (potato late)
8. Bacterial-Resistant Crops (e.g., crops under Early flowering - etc., etc.)
9. Here Now

1. Herbicide-Resistant Crops (corn, soybean, canola)
2. Insect-Resistant Crops (corn, cotton)
3. Viral-Resistant Crops (papaya, potato)
Regulating GMOs

Animal Biotechnology - Science-Based Concerns
National Research Council
National Academies Press, 2002

Environmental Effects of Transgenic Plants: Scope and Adequacy of Regulation
National Research Council
National Academies Press, 2002
OPPOSITION TO GENETICALLY MODIFIED PLANTS

1. Ideology - Don't change Nature (Politics)
2. Anti-Technology - Symbol for technology being central in western society - Anti-Science
3. Anti-Market - Globalization - Industry taking over food supply
4. Protectionism - American Agro companies outcompeting European Agro companies - First generation "losers"
5. Anti-Eugenics - Experience in WWII
6. Organic Growers
7. Ecology - Genetically Modified Crops/Plants outcompeting "natural" species
8. Do Not Need in West - Personal Control/Liberty - Labeling
9. No Obvious Consumer Benefit
10. Easy Target for Anti-gene Technology
11. Lack of Confidence in Government - No FDA, EPA, USDA - Symbol of all "disasters" - BSE, Bovine, etc.