Useful Bacterial Metabolites that can be Engineered

Table 5.1 Examples of Primary and Secondary Metabolites Produced by Fermentation

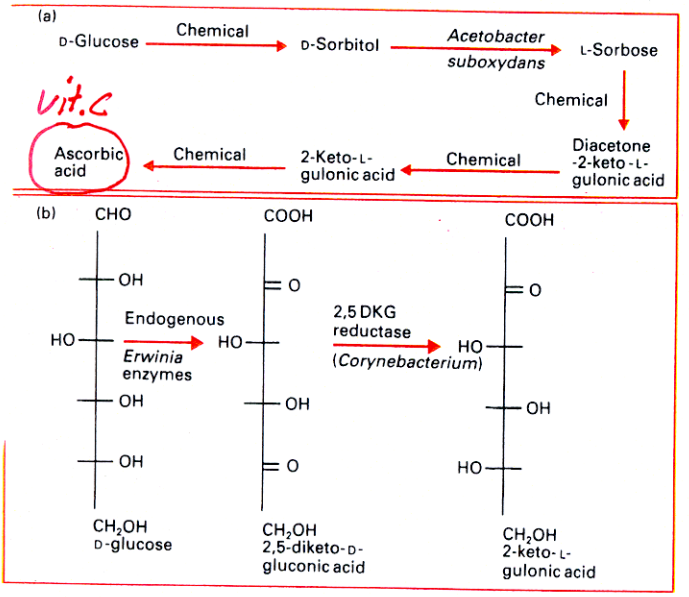
Primary Metabolites	Secondary Metabolites
Amino acids	Antibiotics
Vitamins	Pigments
Nucleotides	Toxins
Polysaccharides	Alkaloids
Ethanol	Many active pharmacological compounds (e.g., the immunosuppressor cyclosporin, hypotensive compound dopastin)
Acetone	
Butanol	
Lactic acid	

Organic Chemical	Microbial Sources	Selected Uses
Acetic acid	*Acetobacter*	Industrial solvent and intermediate for many organic chemicals, food acidulant
Acetone	*Clostridium*	Industrial solvent and intermediate for many organic chemicals
Acrylic acid	*Bacillus*	Industrial intermediate for plastics
Butanol	*Clostridium*	Industrial solvent and intermediate for many organic chemicals
2,3-Butanediol	*Aerobacter, Bacillus*	Intermediate for synthetic rubber manufacture, plastics and antifreeze
Ethanol	*Saccharomyces*	Industrial solvent, intermediate for vinegar, esters and ethers, beverages
Formic acid	*Aspergillus*	Textile dyeing, leather treatment, electroplating, rubber manufacture
Fumaric acid	*Rhizopus*	Intermediate for synthetic resins, dyeing, acidulant, antioxidant
Glycerol	*Saccharomyces*	Solvent, plasticizer, sweetener, explosives manufacture, printing, cosmetics, soaps, antifreeze
Glycolic acid	*Aspergillus*	Textile processing, pH control, adhesives, cleaners
Isopropanol	*Clostridium*	Industrial solvent, cosmetic preparations, antifreeze, inks
Lactic acid	*Lactobacillus, Streptococcus*	Food acidulant, dyeing, intermediate for lactates, leather treatment
Methylethyl ketone	*Chlamydomonas*	Industrial solvent, intermediate for explosives and synthetic resins
Oxalic acid	*Aspergillus*	Printing and dyeing, bleaching agent, cleaner, reducing agent
Propylene glycol	*Bacillus*	Antifreeze, solvent, synthetic resin manufacture, mold inhibitor
Succinic acid	*Rhizopus*	Manufacture of lacquers, dyes and esters for perfumes

Optimize using genetic Engineering

MICROBES CAN BE ENGINEERED TO PRODUCE IMPORTANT MOLECULES that were Made Previous By Chemical Reactions

e.g.: → VITAMIN C synthesis



Chemical

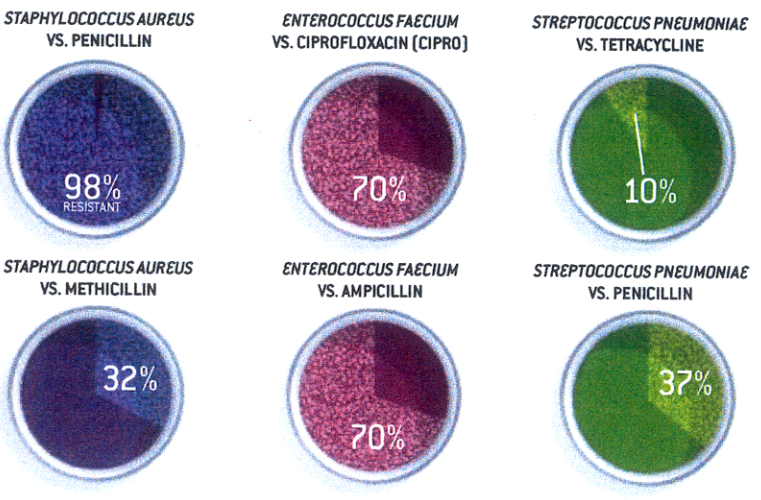
Biology-based

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.

Antibiotic Resistance is a major problem

RISING RESISTANCE

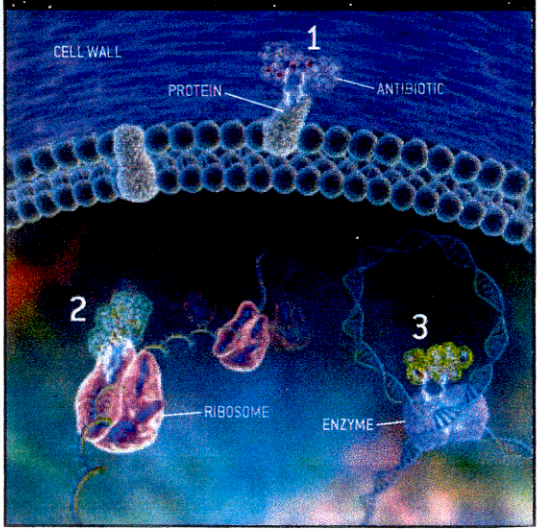
MANY ANTIBIOTICS are no longer effective against certain strains of bacteria, as these examples—collected from different hospitals in the late 1990s—show. One strain of *Staphylococcus aureus* found in Korea, for instance, is 98 percent resistant to penicillin (*top left*); another, found in the U.S.,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



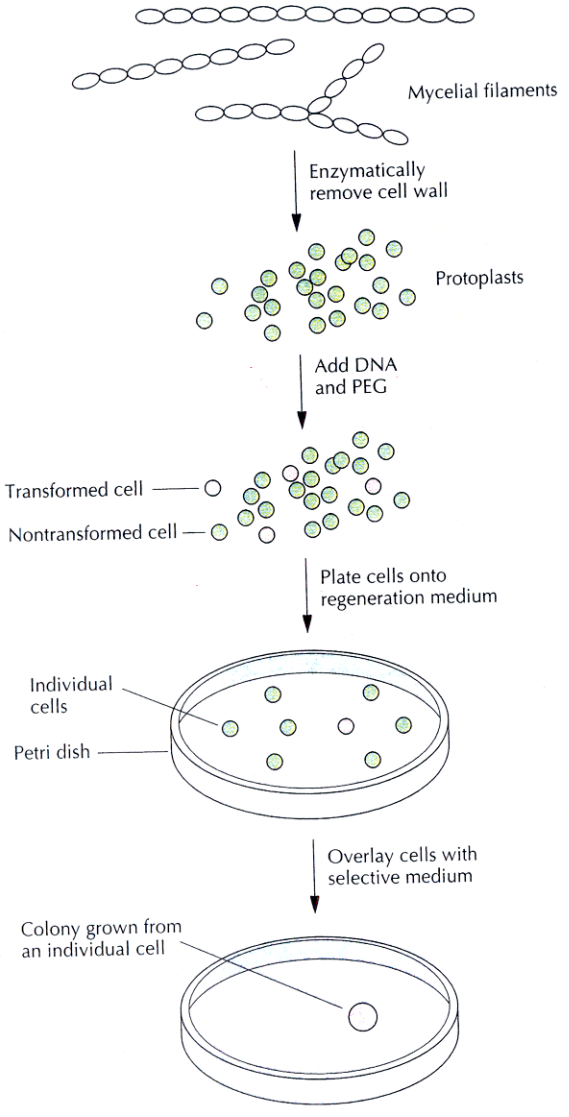
How Antibiotics Work

ANTIBIOTICS AT WORK

EXISTING ANTIBIOTICS fight infections by preventing bacteria from making essential substances. Vancomycin and ß-lactam antibiotics interfere with synthesis of the cell wall (*1*). Erythromycin and tetracycline disrupt ribosomes that make proteins (*2*). Quinolone antibiotics inhibit enzymes involved in replicating DNA (*3*), and sulfonamide antibiotics also interfere with DNA synthesis (*not shown*).



Novel Antibiotics CAN BE Engineered in Bacteria



Streptomyces → Streptomycin!

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.

BACTERIA CAN BE Engineered TO HAVE NOVEL Degradative PATHWAYS FOR BIOREMEDIATION

PLASMIDS

Table 13.1 *Pseudomonas* plasmids, their degradative pathways, and their sizes

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

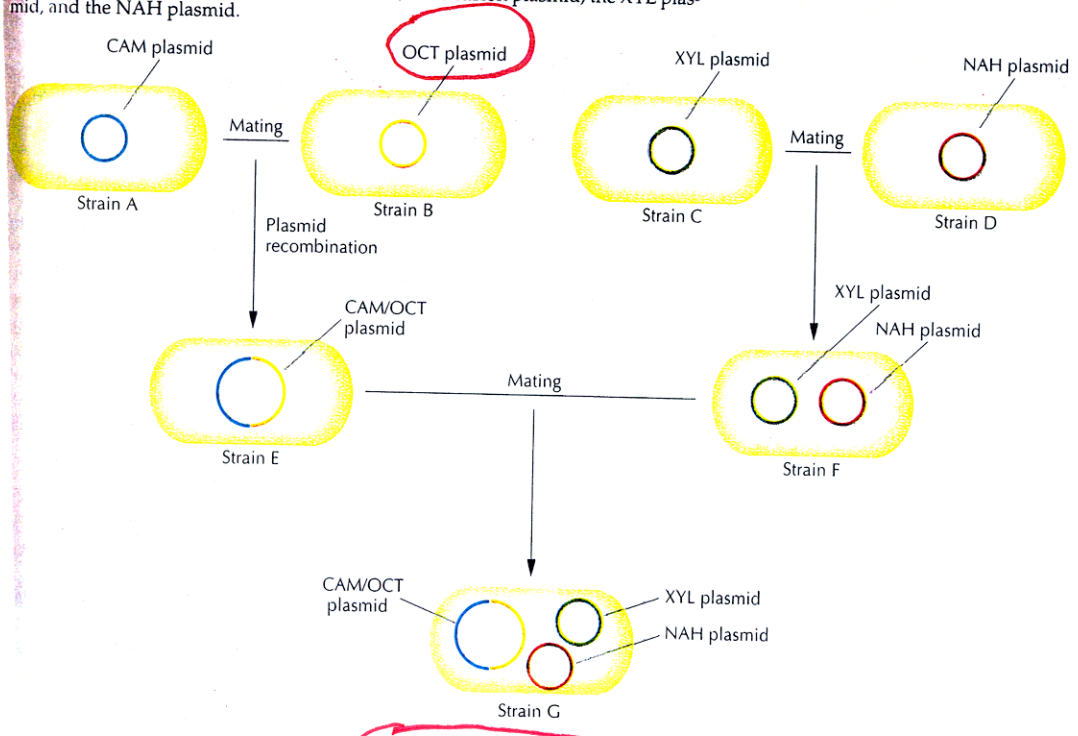
Adapted from Cork and Krueger, *Adv. Appl. Microbiol.* 36:1–66, 1991.

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.

PSEUDOMONAS



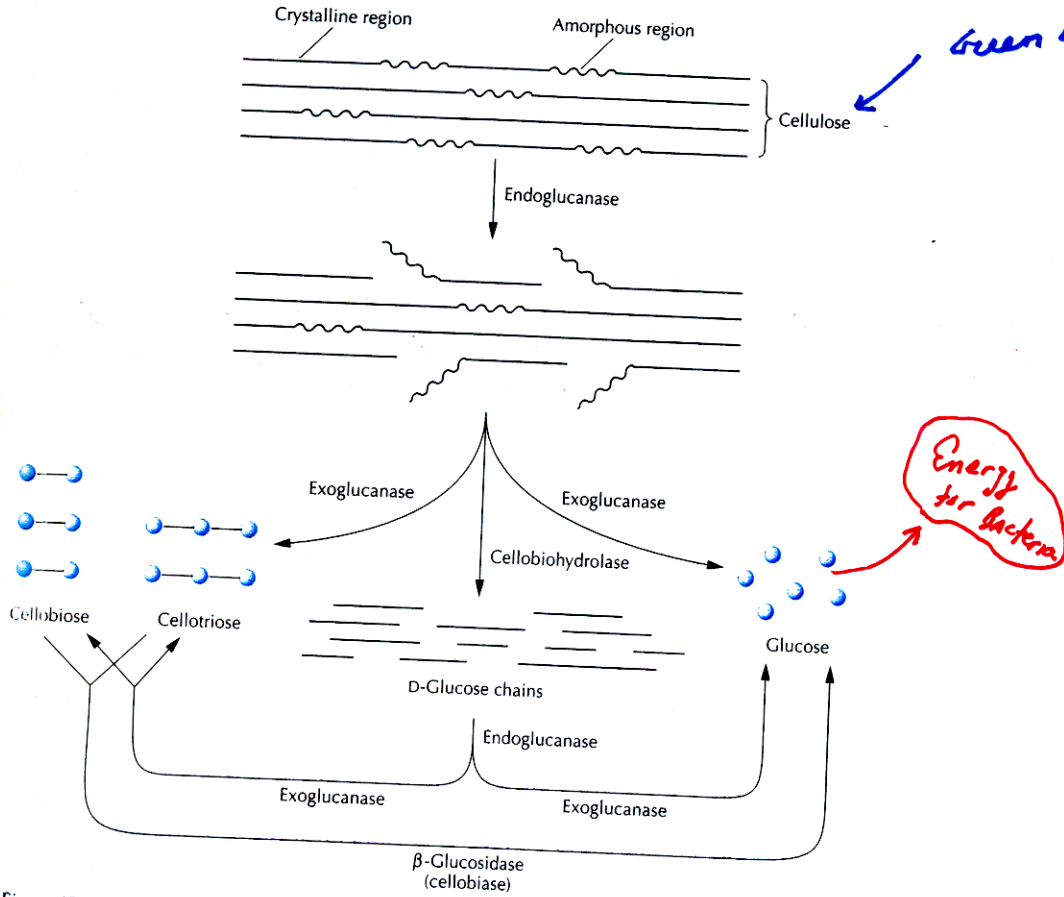
A LANDMARK DECISION

CHAKRABARTY US PATENT 4,259,444 1981
genetically engineered MICROORGANISMS ARE "inventions"

LIFE CAN BE PATENTED!

BACTERIA CAN BE Engineered To degrade BIOMASS WASTE Products

WASTE containing cellulose



Green waste!

Energy for bacteria

Figure 13.15 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 cellobiohydrolase(s). The degradation of cellulose is completed when the cellobiose and cellotriose are converted to glucose by β-glucosidase.

Agriculture, Timber Processing, Human Activities:
e.g.) plants left after harvests, animal manure with grasses, municipal waste paper, cotton left-overs, hay, etc

Bacteria

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

Hassan Brim[1], Sara C. McFarlan[2], James K. Fredrickson[3], Kenneth W. Minton[1], Min Zhai[1], Lawrence P. Wackett[2], and Michael J. Daly[1]*

RESEARCH ARTICLES

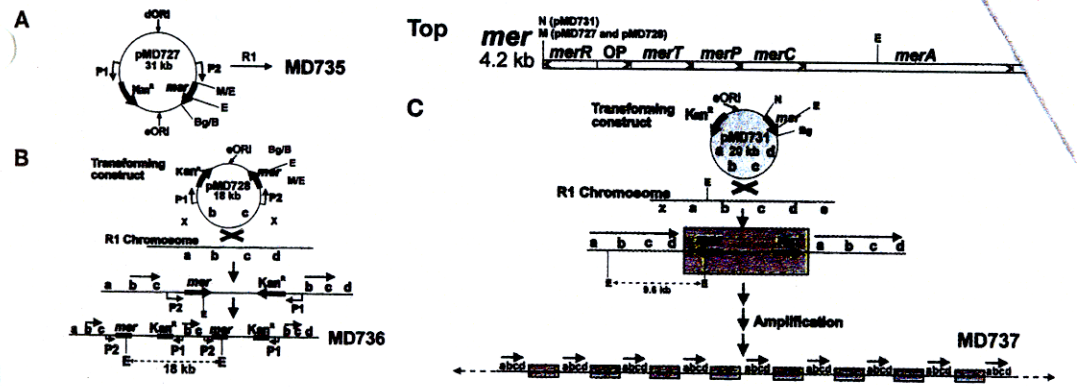


Figure 1. Plasmid and chromosomal maps. (A) 4.2-kb *mer* operon of pBD7[24] encodes six proteins: MerR, activation/repression of the *mer* operon; MerT, mercuric ion transport protein; MerP, periplasmic mercuric ion binding protein; MerC, transmembrane protein; MerA, mercuric reductase; and MerD, putative secondary regulatory protein. OP, operator/ promoter sequence; M, *MfeI*; N, *NcoI*; E, *EcoRI*; Bg, *BglII*. (A) pMD727 was transformed into *D. radiodurans* strain R1 by selection with kanamycin (Kan), giving MD735. dORI, deinococcal origin of replication[18]; eORI, *E. coli* origin of replication[18]. P1 and P2 are two different constitutive deinococcal promoters[21,25]. Kan^R, kanamycin resistance gene *aphA*; *mer*, mercury operon. Bg/B, *BglII*/*BamHI* fusion; M/E, *MfeI*/*EcoRI* fusion. (B) pMD728 was transformed into strain R1 with *Km* selection, giving MD736. Two rounds of recombinative duplication are illustrated, yielding two vector copies on a chromosome. bc, duplicated chromosomal target sequence; X, *Xba*1; all other abbreviations and symbols, as in A. (C) pMD731 was transformed into strain R1 with *Km* selection, giving MD737. Several rounds of recombinative duplication are illustrated, yielding many insertions per chromosome. abcd, duplicated chromosomal target sequence; all other abbreviations and symbols, as in A and B above.

Hg(II) → Hg(0) → Vapor

Metal Chelate In Soil

Engineering Bacteria for the Benefit of Agriculture

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!

TOP 20 Crops in California + USA

Rank	Crops in California[a]	Area planted (106 hectares)
1	Alfalfa	0.38
2	**Cotton lint, all**	0.34
3	Grapes, all	0.32
4	**Rice**	0.20
5	Almond (shelled)	0.19
6	Wheat, all	0.18
7	**Tomato, all**	0.15
8	Lettuce, all	0.09
9	**Maize, grain**	0.082
10	Oranges, all	0.081
11	Walnuts	0.077
12	Beans, dry	0.053
13	Barley	0.05
14	Broccoli	0.05
15	**Sugar beets**	0.043
16	Carrots	0.037
17	Prunes, dry	0.034
18	Pistachios	0.029
19	Sunflower	
20	**Canola**	

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

[a] Estimated area of production in California for 2000 (California Department of Food and Agriculture Resource Directory)

[b] 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

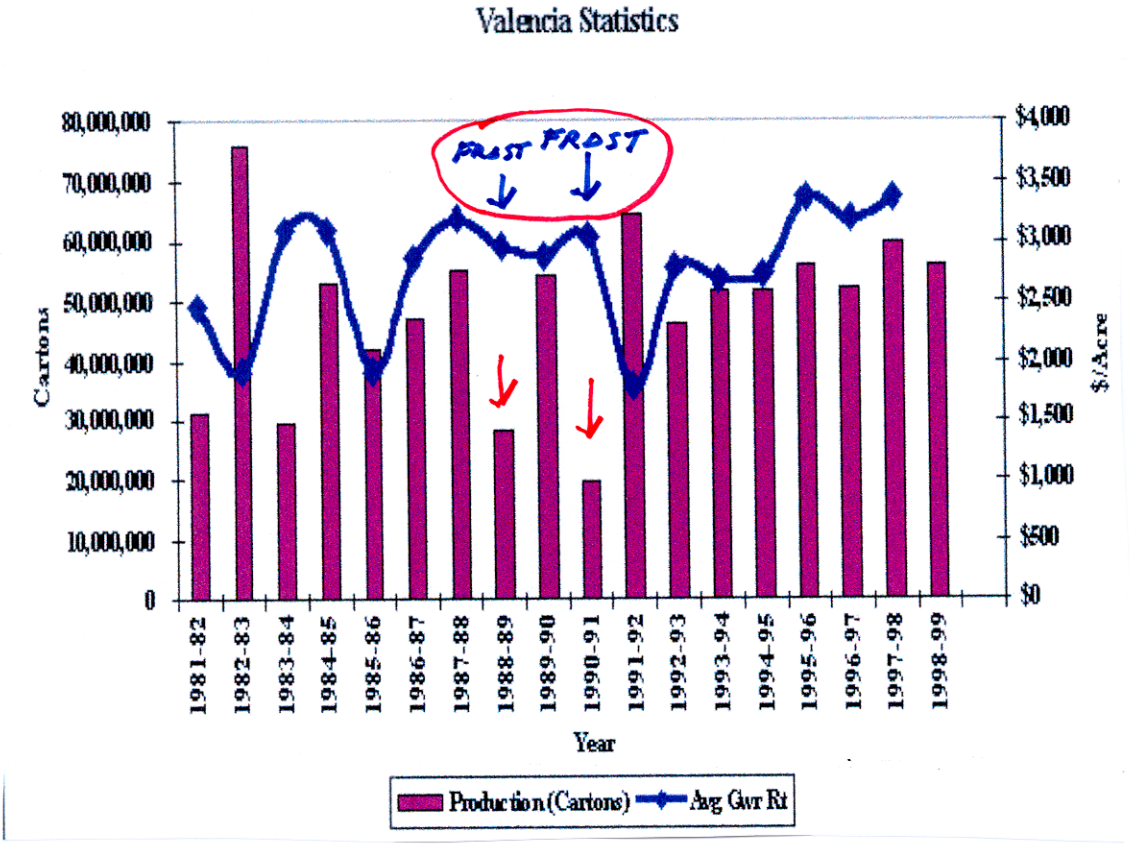
Total Fruit Exports – $1.6 Billion supporting
$3.2 Billion in Economic Activity
& 33,000 jobs!

CALIFORNIA CITRUS

TOTAL FARM VALUE 2001 – $30 billion

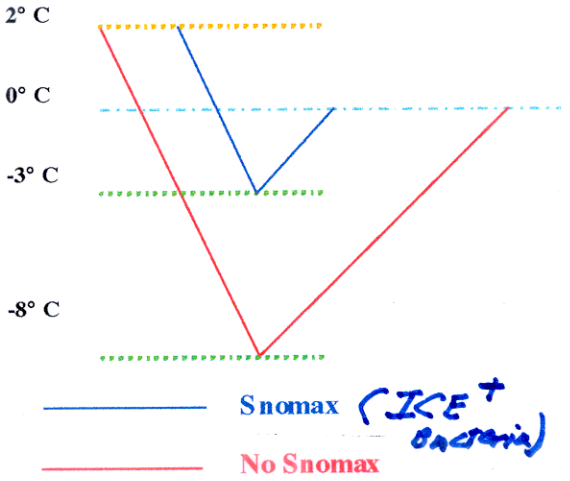
Citrus Value – $1.5 billion

CITRUS LOSS DUE TO FREEZING



ICE FORMATION

Water cooling and freezing



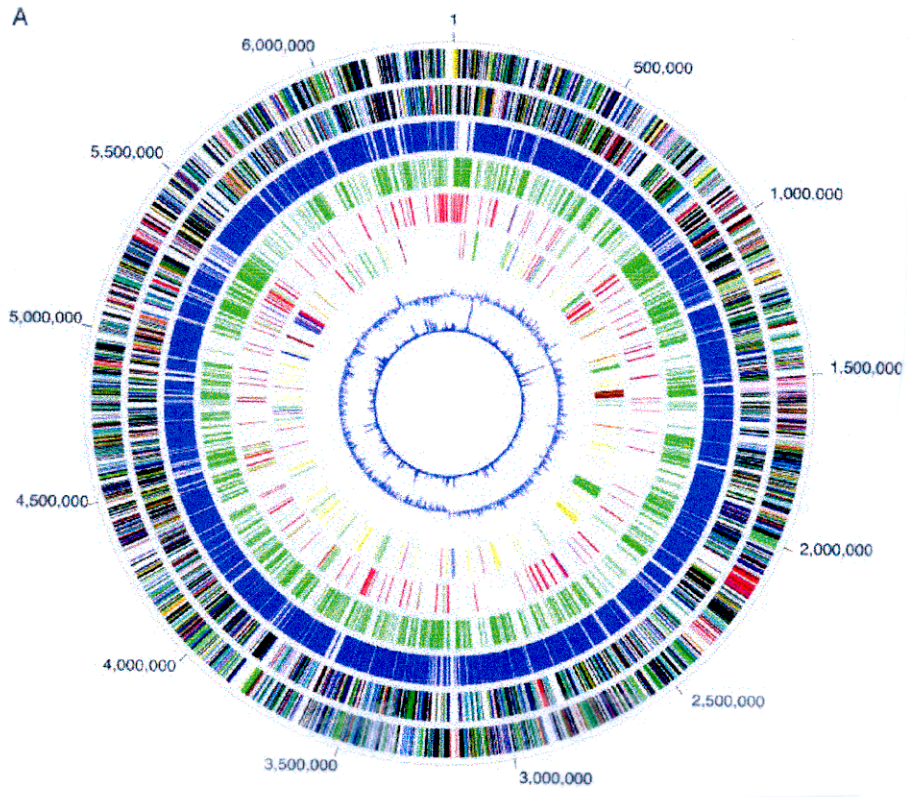
FREEZING REQUIRES Supercooling & Ice crystal nucleation

Pseudomonas Ice+ Bacteria can do This

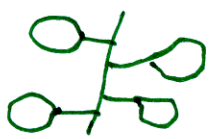
NOTE: ICE+ Bacteria Nucleate Supercooled water at a Higher Temperature to cause Freezing!

Pseudomonas syringae

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



Genome = 6.7 Mb

colonizes  Plants - lives on leaves

Some strains harmful - Some not -

Box 14·1 Bacterial ice nucleation [5]

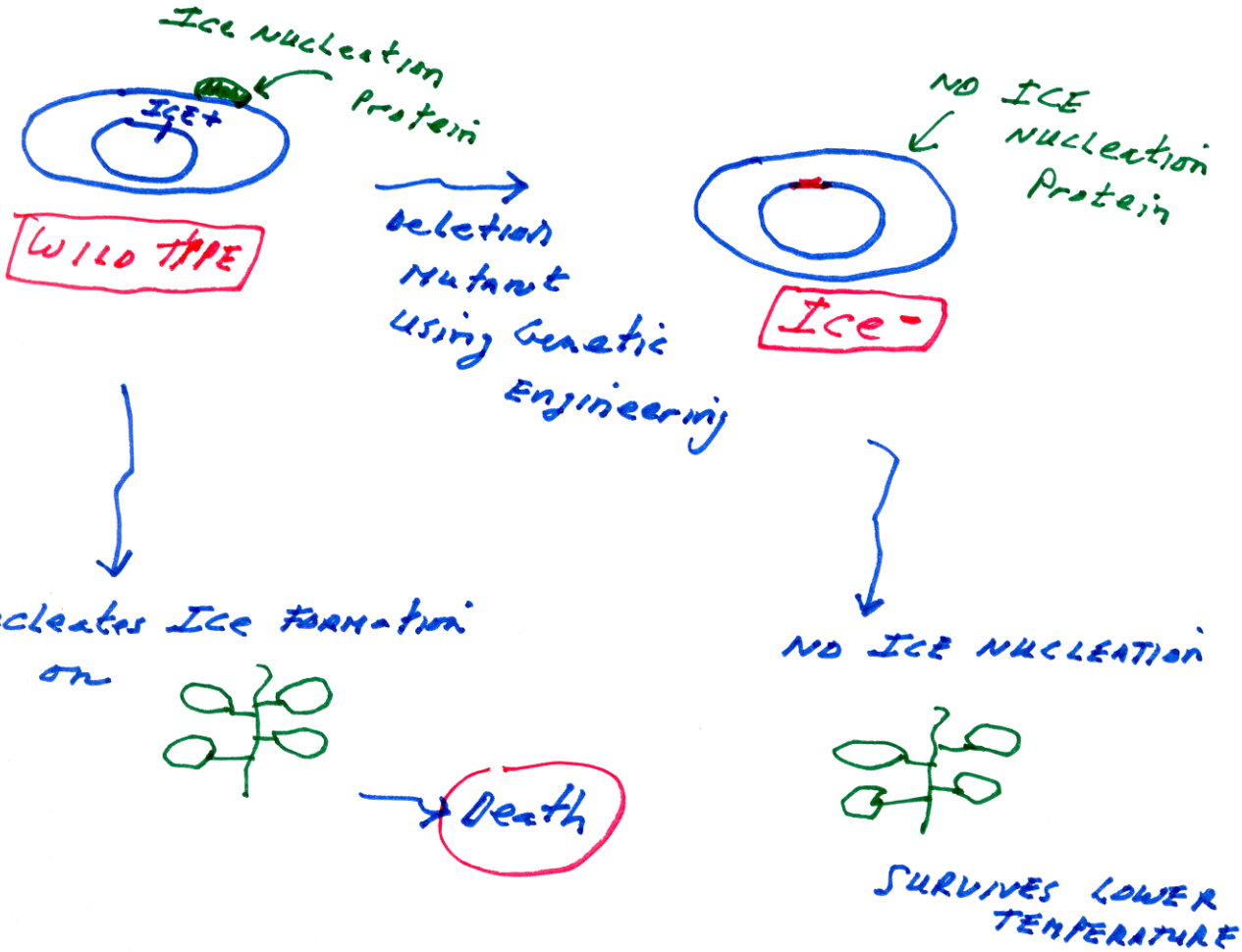
Frost-sensitive plants cannot tolerate ice crystal formation within their tissues. Ice crystals within sensitive plant tissues propagate rapidly, both intercellularly and intracellularly, causing mechanical breakdown of plant tissue and subsequent death. Many liquids, including water, do not invariably 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°C to −20°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°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°C to −5°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.

1: P06620. Ice nucleation pr...[gi:124117] BLink, Links

```
LOCUS       P06620                  1200 aa            linear   BCT 15-SEP-2003
DEFINITION  Ice nucleation protein.
ACCESSION   P06620
VERSION     P06620  GI:124117
DBSOURCE    swissprot: locus ICEN_PSESY, accession P06620;
            class: standard.
            created: Jan 1, 1988.
            sequence updated: Jan 1, 1988.
            annotation updated: Sep 15, 2003.
            xrefs: gi: 45828, gi: 45829, gi: 2144939, gi: 443043, gi: 443044
            xrefs (non-sequence databases): InterProIPR000258, PfamPF00818,
            PRINTSPR00327, PROSITEPS00314
KEYWORDS    Ice nucleation; Repeat; Outer membrane; 3D-structure.
SOURCE      Pseudomonas syringae pv. syringae
  ORGANISM  Pseudomonas syringae pv. syringae
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (residues 1 to 1200)
  AUTHORS   Green,R.L. and Warren,G.J.
  TITLE     Physical and functional repetition in a bacterial ice nucleation
            gene
  JOURNAL   Nature 317, 645-648 (1985)
  REMARK    SEQUENCE FROM N.A.
            STRAIN=S203
REFERENCE   2  (residues 1 to 1200)
  AUTHORS   Lindow,S.E., Lahue,E., Govindarajan,A.G., Panopoulos,N.J. and
            Gies,D.
  TITLE     Localization of ice nucleation activity and the iceC gene product
            in Pseudomonas syringae and Escherichia coli
  JOURNAL   Mol. Plant Microbe Interact. 2 (5), 262-272 (1989)
  MEDLINE   92297969
   PUBMED   2520825
  REMARK    SUBCELLULAR LOCATION.
REFERENCE   3  (residues 1 to 1200)
  AUTHORS   Kajava,A.V. and Lindow,S.E.
  TITLE     A model of the three-dimensional structure of ice nucleation
            proteins
  JOURNAL   J. Mol. Biol. 232 (3), 709-717 (1993)
  MEDLINE   93360260
   PUBMED   8355267
  REMARK    3D-STRUCTURE MODELING OF 490-535.
COMMENT     ---------------------------------------------------------------------
            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
            ---------------------------------------------------------------------.
            [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-T-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



GROWTH OF WILD-TYPE AND DELETED PSEUDOMONAS STRAINS

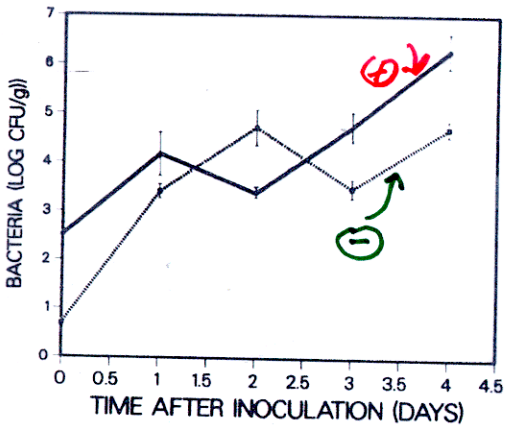


FIG. 2. Population size of *P. syringae* TLP2 (○——○) and TLP2del1 (■- - -■) as a function of time on wet potato leaves. Potato leaves were inoculated with an aqueous suspension of ca. 10^4 CFU of each bacterial strain per ml and placed in a mist chamber at 24°C. Leaves were harvested and bacterial populations 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.

NO SIGNIFICANT GROWTH OR SURVIVAL DIFFERENCES

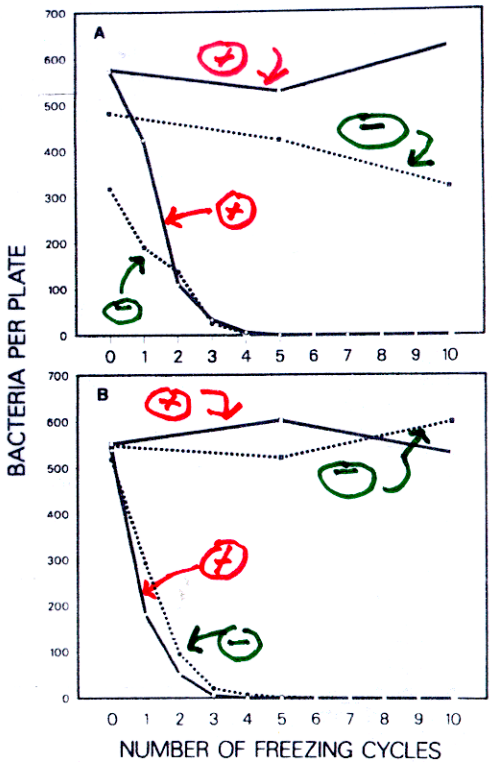


FIG. 4. Survival of Ice$^+$ parental strains (○——○) and Ice$^-$ deletion mutants (●- - -●) of *P. syringae* TLP2 (A) and Cit7 (B) during successive freeze-thaw cycles. Bacterial strains were suspended in tubes containing 100 mM potassium phosphate buffer (pH 7.0) containing 0.1% peptone at a concentration 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 incubated 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.

Hypothesis: Releasing Ice© Pseudomonas on crops should protect them from freezing!

SAVE CROPS & BILLIONS OF DOLLARS of CROP LOSS!

Players: Steve Lindow, UC Berkeley

Advanced Genetic Sciences (AGS)

USDA

EPA

NIH RAC

Foundation for Economic Trends - Jeremy Rifkind et al.

Start: 1983 (22 years ago!)

Finish: 1987

A LONG FIVE YEAR PROCESS

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 coded 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 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 fate, 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

The RESULTS

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 DELITEROUS 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.

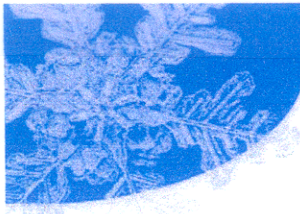
THE OUTCOME
2004

FROST CONTINUES TO CAUSE MAJOR LOSSES TO CROPS WORLD-WIDE

ICE⊖ BACTERIA HAS BECOME A HISTORICAL FOOTNOTE – LONG FORGOTTEN & NOT USED. Why? Regulatory costs, partly by it being classified as a pesticide

But... ICE⊕ Bacteria found a NICHE ON THE SKI SLOPES!

IRONY?

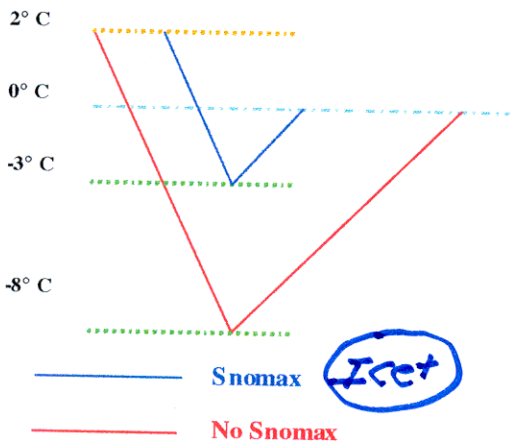


What Exactly Is Snomax And How Does It Work?




Snomax®
YORK INTERNATIONAL

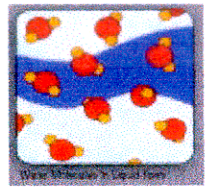
Water cooling and freezing



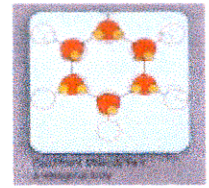
2°C = water temperature
-3°C = freezing point for water with Snomax
-8°C = freezing point for water without Snomax

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



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 and environment

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 Biolink 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 controled 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 m^3 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!

Alternatives ARE ON THE WAY!

Mendel Biotechnology

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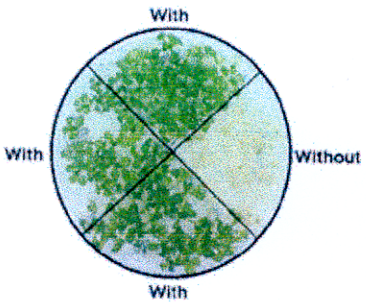
- WeatherGard™ Genes
- Other Products

WeatherGard™ Genes



WeatherGard™ Genes

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.

Make a Freezing Tolerant Plant!

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.

Wake up, and Smell the Coffee

By Henry Miller
***The Washington Times* (June 30, 1997)**

Californians are in crisis mode. Is the cause drive-by shootings, tornados, 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 (dubbed "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 "pesticide." (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.

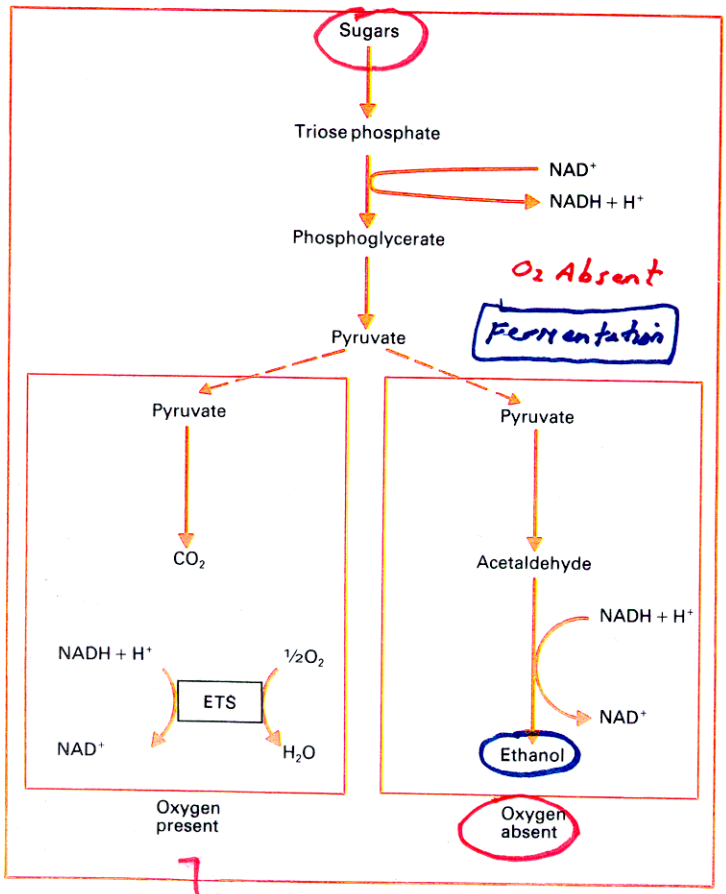
Henry Miller is a senior research fellow at the Hoover Institution and author of "Policy Controversy in Biotechnology: An Insider's View."

USING YEAST AS FACTORIES AND "CATALYSTS"

Table 36.1 Fungi

Phylum	Typical Examples		Key Characteristics	Approximate Number of Living Species
Ascomycota	Yeasts, truffles, morels		Develop by sexual means; ascospores are formed inside a sac called an ascus; asexual reproduction is also common	32,000
Imperfect fungi	*Aspergillus*, *Penicillium*		Sexual reproduction has not been observed; most are thought to be ascomycetes that have lost the ability to reproduce sexually	17,000
Basidiomycota	Mushrooms, toadstools, rusts		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	22,000
Zygomycota	*Rhizopus* (black bread mold)		Develop sexually and asexually; multinucleate 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	1050

What are fungi & yeasts?



USING YEAST TO MAKE RECOMBINANT PROTEINS

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
- α_1 antitrypsin
- Blood coagulation factor XIIIa
- Hirudin
- Human growth factor
- Human serum albumin

Advantages over Bacteria?

Vectors?

Switches?

USING YEAST TO MAKE ALCOHOLIC BEVERAGES

Table 6.5 The origins of the different kinds of alcoholic beverages.

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

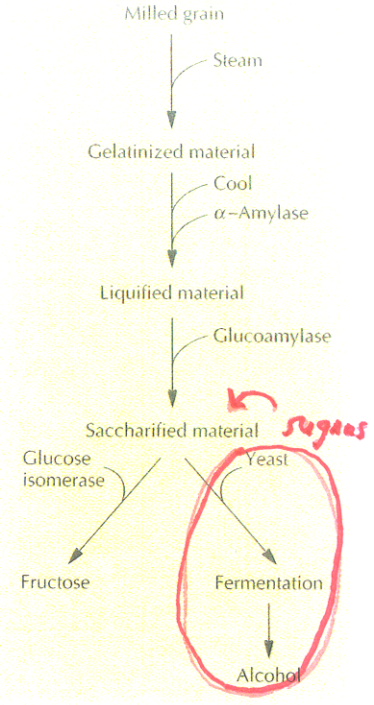


Figure 13.10 Industrial production of fructose and alcohol from starch.