further investigation of ascorbate metabolism could illuminate how its biosynthesis evolved and perhaps even contribute to evidence on the evolutionary relationships of eukaryotes.

Although Agius *et al*. have shown that it is feasible to engineer increased ascorbate, in the current economic climate, there is apparently little commercial interest in genetically modified crops with enhanced ascorbate, largely because most people easily achieve the recommended intake. However, the ability to engineer ascorbate may help us understand, along with existing resources, such as the low ascorbate *vtc* mutants[3,5], how ascorbate helps to control reactive oxygen species and increases stress resistance. Other approaches to elucidating the pathways and control of ascorbate biosynthesis, including use of *A. thaliana* genomic resources (for example, natural variation and activation tagging) and identification of transport mechanisms[11] may also provide information. Another application for novel enzymes is the development of fermentation-based processes to manufacture ascorbate[12]. However, the low cost of established processes for ascorbate manufacture means that new processes would have to be highly efficient to compete.

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Pseudomonas putida—a versatile biocatalyst

Genomic analysis of *Pseudomonas putida* sheds light on metabolic pathways that may be exploited for a variety of biotechnological applications.

Lawrence P. Wackett

Chemists have been successful in constructing millions of new organic chemicals, over 60,000 of which are currently sold worldwide. When organic chemicals enter the environment, whether by accident or design, they often are degraded by microbes. Over the past century, microbes have been isolated from the environment to study their degradative metabolism in the laboratory. More have turned out to be *Pseudomonas putida* than any other single bacterial species. Additionally, the novel reactions used by *Pseudomonas* strains to degrade wastes have, in some cases, been harnessed by biotechnology to synthesize specialty chemicals. For example, Nitto Chemical Company (Tokyo, Japan) has found a way to short-circuit the biodegradative pathway for transforming acrylonitrile to acrylate (Fig. 1), thus developing a large-scale industrial process to make the commodity chemical acrylonitrile[1]. Revealing the underlying molecular basis of biotransformations by *P. putida* will contribute greatly to its potential use in novel biotechnological processes. In this context, it is exciting to see the special December 2002 issue of *Environmental Microbiology* that has been devoted to the functional genomic analysis of *Pseudomonas* species.

Functional genomic analysis of *P. putida* has been made possible by the completion of the much-anticipated genome sequencing project that has been jointly supervised by The Institute for Genomic Research (Rockville, MD) in the United States and the Medizinische Hochschule Hannover, the Gesellschaft für Biotechnologische Forschung (Braunschweig), the Deutsches Krebsforschungszentrum (Heidelberg), and Qiagen (Hilden) in Germany. Highlights of the *Environmental Microbiology* special issue are an analysis by Karen Nelson and her coworkers of the complete genome sequence of *P. putida* K2440 (ref. 2) and additional reports that constitute a functional genomic analysis of the same organism[3–6]. The special issue also includes editorials that provide a historical perspective for understanding the worldwide interest in strain *P. putida* K2440.

Throughout the past century, *Pseudomonas* species have figured prominently in efforts to unravel how microbes recycle disparate organic molecules in the environment. In 1926, der Dooren de Jong reported that a *Pseudomonas* species would grow on 80 different organic compounds[7]. In 1955, a *Pseudomonas* species was the source of a newly discovered class of enzymes known as oxygenases[8]. Since 1955, hundreds of oxygenases have been identified and found to be prominent in the oxidation of thousands of environmental chemicals, including aromatic hydrocarbons. Of the 527 enzymes currently cataloged in the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD, http://umbbd.ahc.umn.edu), over 30% are oxygenases and many of them have been identified in *Pseudomonas* strains. Of those *Pseudomonas* strains identified to the species level in the UM-BBD, 47% are *P. putida*. Consistent with these data uncovered during research that spans decades, the current genome annotation for *P. putida* K2440 identified 33 putative genes encoding oxygenases[2].

P. putida strains have been described that catabolize natural products (vanillin, α-pinene, limonene, mandelate, camphor, and adamantanone) and industrial compounds (bromoxynil, styrene, methyl-*tert*-butyl ether (MTBE), trichloroethylene, and nitroglycerin). Some of these compounds are shown in the metabolic map of Figure 1, which illustrates the diverse reactions that pseudomonads use to funnel broad compound classes into trunk metabolism that is more commonplace amongst soil bacteria. Genes encoding catabolic pathways for protocatechuate, catechol, *p*-hydroxybenzoate, vanillin, and arylsulfonates (Fig. 1) have been identified in the *P. putida* K2440 genome[2,3].

Enzymes for the metabolism of toluene, xylenes, and methylbenzoates, were once encoded by the complete genetic material of the original *P. putida* K2440 strain, known as strain mt-2, that contained the pWWO (TOL) plasmid. The complete sequence of the TOL plasmid has been described in the same issue of *Environmental Microbiology*[6]. Environmental *Pseudomonas* strains typically contain multiple plasmids; for example, *Pseudomonas* sp. ADP contains five or more plasmids that collectively account for more than 1 Mbp of DNA[9]. Considering that these plasmids often contain catabolic genes for camphor, octane, salicylate, atrazine, and other compounds, they impart a significant addition to the host organism's metabolic network. The lack of plasmids in present-day *P. putida* K2440 reflects its long habitation in the laboratory where plasmids that carry primarily catabolic

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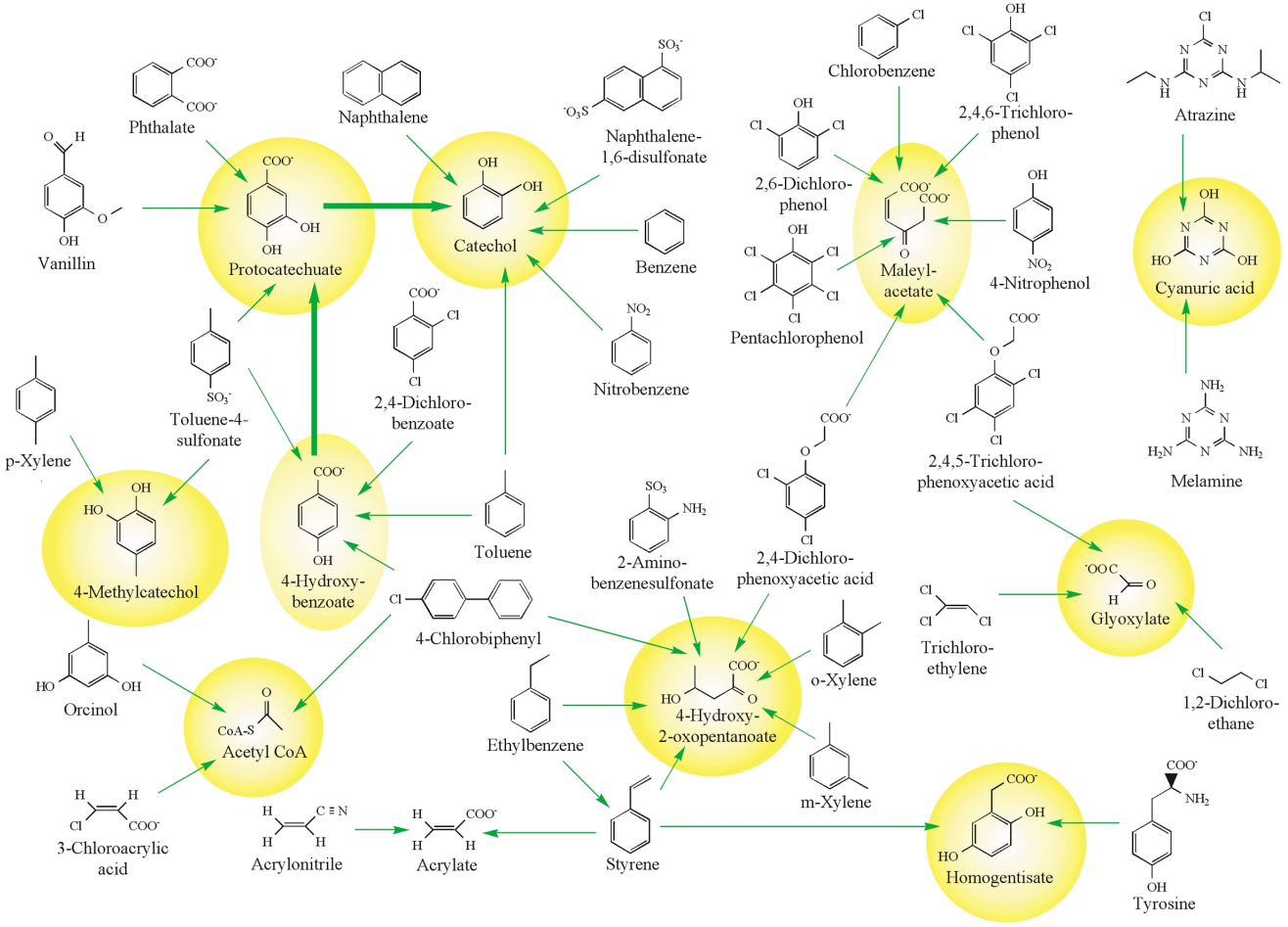

Figure 1. A composite metabolic network showing the transformation of industrial and natural product chemicals by *Pseudomonas* species. The yellow circles surround metabolites that funnel into common intermediary metabolism.

genes are often readily lost.

Genome sequencing typically provides insights into an organism's environmental niche, and this is readily apparent with the annotation of the *P. putida* genome. The metabolism of aromatic acids can now be viewed against the backdrop of this organism's facility to associate with the roots of plants. Identified genes that could contribute to the ability of *P. putida* K2440 to live in association with plant roots include those for cellulose biosynthesis, the biosynthesis of complex surface carbohydrates, and the catabolism of opines and other secreted plant products. Although *P. putida* K2440 may interact with plants in nature, it appears to be lacking most of the requisite genes for establishing a pathogenic relationship with plants. Unlike *Pseudomonas aeruginosa*, *P. putida* is not considered a pathogen, for either plants or animals. This adds to its potential usefulness for industrial and environmental applications. Further details of the sequence annotation are readily available on the World Wide Web at the TIGR Comprehensive Microbial Resource website[10]. This should facilitate data mining by interested academic and industrial researchers.

The present report of the genome sequence, coupled with an extensive analysis of *P. putida*[2–6], represents an important accomplishment in a series of sequencing projects for bacteria that are critical for the recycling of organic carbon on this planet, and include: *Burkholderia cepacia* J2315, *Pseudomonas fluorescens* Pf0-01, *Pseudomonas fluorescens* SB W25, *Rhodococcus* sp. I24, *Rhodococcus* sp. RHA1, and *Sphingomonas aromaticivorans* F199. The further integration of genomic, metabolic, and environmental information is ushering in an age of metabolic prediction and this promises to transform several human endeavors.

First, our continuing efforts to safeguard the environment will benefit by our ability to predict the fate of chemicals that have been synthesized specifically to enter the environment. Thus, herbicides and insecticides will be designed partly with biodegradability in mind, and pathways for their biodegradation will be predicted based on genomic and metabolic knowledge. Second, increased information on the range of possible biocatalytic reactions, and the genes that encode the enzymes, will be a boon for biotechnology. Currently, organic chemistry is used predominantly in the manufacture of industrial chemicals. This is due to the ready availability of synthetic reagents that make possible the manufacture of millions of organic chemicals. We can anticipate a future in which novel microbial genes and enzymes will be as readily available as synthetic reagents. This will lead to the use of cheap biocatalysts and provide a competitive system for the synthesis of chemicals currently made by industry and the development of new products that are inaccessible by conventional organic synthesis.

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Designer 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.

Costas N. Karatzas

Transgenic dairy animals producing milks with altered composition, other than pharmaceutical proteins, are becoming a reality. Designer milks, specialty milks, or humanized milks may be competing in the next ten years to capture part of the global dairy product market worth $400 billion annually. The concept of modifying milk composition (Table 1) by augmenting the protein content of milk through increased casein gene dosage in the cow genome has been postulated for years[1–3]. Now, Brophy *et al.*[4] have reduced this concept to practice, generating eight transgenic cows—through a combination of genetic engineering and nuclear transfer techniques[4,5]—that produce in their milk elevated levels of β- and κ-casein proteins. The use of the nuclear transfer procedure allowed selection of gender and elite genetic background[4,5]. DNA transgene integration was random, similar to pronuclear microinjection gene transfer experiments, and therefore the authors produced a few transgenic lines in order to select the best-expressing animals[4].

Milk, a "nearly perfect food" because of its balanced protein, fat, carbohydrate, and mineral content, represents a fundamental dietary ingredient of many societies. Intensive crossbreeding strategies, nutritional management, and quantitative genetics have resulted in a steady improvement in milk yield, but have not produced major changes in milk protein composition. The world production of fluid milk in 2001 was 474 gigaliters!

A glass of milk contains 8.0 grams of protein, with caseins comprising 78–80% of this amount[1–3]. Together with fat, the casein proteins are responsible for the characteristic white chalky children's milk "mustache". There are four casein proteins in cow's milk[1,2]: αS1- and β-casein (10 g/l each), αS2-casein (3.7 g/l), and κ-casein (3.5 g/l). One of the properties of caseins is to bind and sequester calcium phosphate and magnesium within spherical particles named casein micelles[1,2]. The outer surface of these is enriched with κ-casein, which is cleaved by chymosin used in cheese-making to destabilize micelles and form the curd[1,2].

But why alter the casein concentration of milk? The percentage of casein in milk determines cheese yield; therefore, a clear incentive for changing milk composition has been the increase in cheese yield[1,2] (see Table 1). An increase of 20% in the content of αS1-casein content of milk would result in an increase of $200 million per year[6]. The milks with increased casein content could also be exploited in the manufacturing of milk protein concentrates (MPC) and casein forms. Edible casein is used in vitamin tablets, instant drinks, and infant formulas. Technical acid caseins are used for paper coatings, cosmetics, button-making, paints, and textile fabrics. In 2000, the United States imported a combined 381.4 million pounds of MPC and casein for a total value of approximately $650 million[7]. High-casein milks, if and when they become available, may influence national milk supply management and international trade standards and regulations[7].

In the present paper, Brophy *et al.*[4] overexpress casein variants, resulting in a 30% increase in the total milk casein or a 13% increase in total milk protein[4]. Although milk was collected from induced lactation during which the mammary gland may not be functioning at maximum capacity, it appeared that the transgenic proteins were produced partially at the expense of the endogenous milk proteins. This competition has also been observed at the mRNA level in transgenic mice overexpressing ovine β-lactoglobulin in their milk[8]. These compensatory events may occur at a post-transcriptional or translational stage or during the passage of proteins through the secretory pathway of the mammary cell[8].

Interestingly, a measurable variation was observed in the concentration of the β- and κ-caseins among the eight transgenic genetic clones[4]. The transgenics generated[4] contain, in addition to embryonic cell–derived nuclear DNA, oocyte-derived mitochondrial DNA and probably also donor cell–derived mitochondrial DNA[9]. As mitochondria are the sites of many metabolic reactions that are an integral part of lactation, it is possible that differences in the genetic bloodline origin of the mitochondria in these transgenic animals

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

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

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