value at each step. The standardization of a degraded signal at each step requires a response of the type shown in Fig. 2b, which is achieved through the use of devices with high gain.

The most prominent defect in novel devices that have been seriously considered as alternatives is a lack of high gain. No device with the high gain of transistors has appeared in the decades since the replacement of the relay and the vacuum tube by transistors. Without high gain close attention to the reproducibility of component parameters is needed, but such reproducibility is difficult to achieve in dense arrays of miniaturized components.

Other desirable features that have been mentioned should not be forgotten: isolation of input and output, ability to perform inversion and to switch both ways in comparable amounts of time so that no separate resetting operation is needed, and a potential for attaining the high packing density needed in high-speed systems.

Finally, the barrier to the introduction of new materials into electronic technology increases each year as conventional silicon technology continues to improve.

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RESEARCH ARTICLE

Production of 2-Keto-L-Gulonate, an Intermediate in L-Ascorbate Synthesis, by a Genetically Modified *Erwinia herbicola*

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William Rastetter, David Estell

The commercialization of recombinant DNA technology has mainly involved the use of convenient microbial or animal cell hosts for the manufacture of foreign proteins. However, the application of this technology in the chemical industry for the production of small molecules has also begun (*1*). We now report the construction of a recombinant "metabolically engineered" bacterial strain that is able to synthesize 2-keto-L-gulonic acid, a key intermediate in the production of L-ascorbic acid (*2*).

At present, most L-ascorbic acid (vitamin C) is produced by a modification of the Reichstein-Grussner synthesis (*3*, *4*), a lengthy and capital-intensive route that involves a microbial fermentation and a series of chemical steps (Fig. 1). The last intermediate in the Reichstein-Grussner synthesis (Fig. 1) is 2-keto-L-gulonic acid (2-KLG), a compound that can easily be converted into L-ascorbic acid via a simple acid- or base-catalyzed cyclization (*2*). A number of organisms from the coryneform group of bacteria (*Corynebacterium*, *Brevibacterium*, and *Arthrobacter*) as well as species of *Micrococcus, Staphylococcus, Pseudomonas, Bacillus*, and *Citrobacter* are able to carry out the microbial conversion of 2,5-diketo-D-gluconic acid (2,5-DKG) into 2-KLG (*5*). Furthermore, a number of species of *Acetobacter, Gluconobacter*, and *Erwinia* can efficiently oxidize D-glucose to 2,5-DKG (*5*, *6*). Thus, 2-KLG can be produced from D-glucose via 2,5-DKG by a cofermentation of appropriate microorganisms from the above two groups (*5*). Sonoyama *et al.* have also published a tandem fermentation process, with the use of mutant strains of *Erwinia* and *Corynebacterium*, for carrying out the glucose to 2-KLG conversion (*7*) (Fig. 2).

Although the tandem fermentation represents a considerable simplification in the route from D-glucose to L-ascorbic acid, our goal was to simplify this process further by combining the relevant traits of both the *Erwinia sp.* and the *Corynebacterium sp.* in a single microorganism. To accomplish this we identified the 2,5-DKG reductase in the *Corynebacterium sp.* (*8*) that was responsible for the conversion of 2,5-DKG into 2-KLG. The gene for this reductase was then cloned and expressed in *Erwinia herbicola*, a bacterium of the family Enterobacteriaceae that is able to convert D-glucose into 2,5-DKG (*8*). The resultant organism is able to convert D-glucose into 2-KLG in a single fermentation (Fig. 2).

Identification, purification, and characterization of the *Corynebacterium sp.* 2,5-DKG reductase. Although the efficient conversion of 2,5-DKG into 2-KLG by whole cells of a *Corynebacterium sp.* mutant strain had been demonstrated (*7*), essentially nothing was known about the

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enzyme responsible for this bioconversion. However, because many of the enzymes that carry out ketone-to-alcohol reductions use a nicotinamide nucleotide cofactor, crude lysates from *Corynebacterium sp.* were assayed for 2,5-DKG reductase activity under a variety of conditions with reduced nicotinamide adenosine diphosphate (NADH) or reduced nicotinamide adenosine triphosphate (NADPH). Several 2,5-DKG reductases were isolated and further characterized to identify the one that produced 2-KLG. This enzyme was purified to homogeneity from the cytosolic fraction of lysed *Corynebacterium sp.* cells by DEAE cellulose ion exchange, Cibacron blue affinity, and high-performance liquid chromatography (TSK gel permeation) (*9*).

The purified 2,5-DKG reductase was found to be a single polypeptide with a mobility of sodium dodecyl sulfate (SDS)–polyacrylamide gels that corresponded to a molecular mass of 34,000 daltons. Molecular mass measurements by gel permeation TSK chromatography yielded a value of 35,000 daltons, suggesting that the native enzyme exists in solution as a monomer. The 2,5-DKG reductase completely and stereoselectively reduced 2,5-DKG to 2-KLG with NADPH as a cofactor; no activity was observed with NADH. The apparent Michaelis constants (K_m) were 15 mM for 2,5-DKG and 34 μM for NADPH, and the V_{max} was 10 μmol/min-mg in 80 mM bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane (*p*H 6.4) at 25°C (*9*). At *p*H 9.0 to 9.5 the observed equilibrium constant K_{eq} (*10*) for the reaction catalyzed by the 2,5-DKG reductase was 5.6×10^{-13}, indicating that 2-KLG formation is thermodynamically highly favored under physiological conditions.

Cloning of the 2,5-DKG reductase gene. Automatic, sequential Edman degradation of the 2,5-DKG reductase revealed the sequence of 40 amino acids from the NH_2-terminus of the protein. Inspection of this sequence revealed no regions of minimal codon degeneracy suitable for the construction of mixed oligonucleotide probes (*11*). Therefore, a cloning strategy was adopted in which long, synthetic sequence DNA probes (*12*) were used. When these long probes are used, it is appropriate to maximize the chances of homology between the probe and the prospective gene sequence by judicious codon choices. Because bacterial DNA's with extreme base compositions are known to have highly biased codon usage patterns (*13*), the thermal melting

Abstract. *A new metabolic pathway has been created in the microorganism* Erwinia herbicola *that gives it the ability to produce 2-keto-L-gulonic acid, an important intermediate in the synthesis of L-ascorbic acid. Initially, a* Corynebacterium *enzyme that could stereoselectively reduce 2,5-diketo-D-gluconic acid to 2-keto-L-gulonic acid was identified and purified. DNA probes based on amino acid sequence information from 2,5-diketo-D-gluconic acid reductase were then used to isolate the gene for this enzyme from a* Corynebacterium *genomic library. The 2,5-diketo-D-gluconic acid reductase coding region was fused to the* Escherichia coli *trp promoter and a synthetic ribosome binding site and was then introduced into* E. herbicola *on a multicopy plasmid.* Erwinia herbicola *naturally produces 2,5-diketo-D-gluconic acid via glucose oxidation, and when recombinant cells expressing the plasmid-encoded reductase were grown in the presence of glucose, 2-keto-L-gulonic acid was made and released into the culture medium. The data demonstrate the feasibility of creating novel in vivo routes for the synthesis of important specialty chemicals by combining useful metabolic traits from diverse sources in a single organism.*

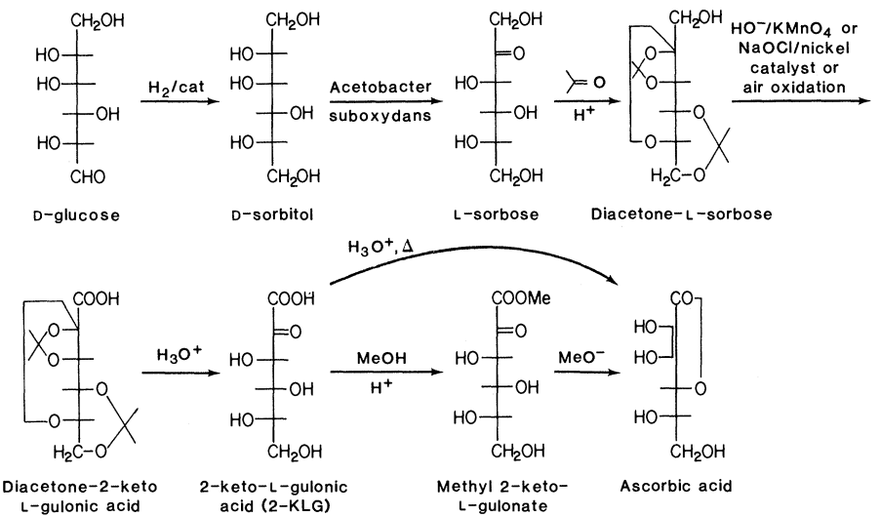

Fig. 1. The Reichstein-Grussner synthesis of L-ascorbic acid (*4*). The oxidation of D-sorbitol to L-sorbose is accomplished by an *Acetobacter suboxydans* fermentation; all the other steps are chemically catalyzed. The curved arrow from 2-keto-L-gulonic acid to L-ascorbic acid represents a nonaqueous acid-catalyzed cyclization (*2*). In the commercial process at present, ascorbic acid is produced by acid cyclization of diacetone-2-keto-L-gulonic acid (*2*).

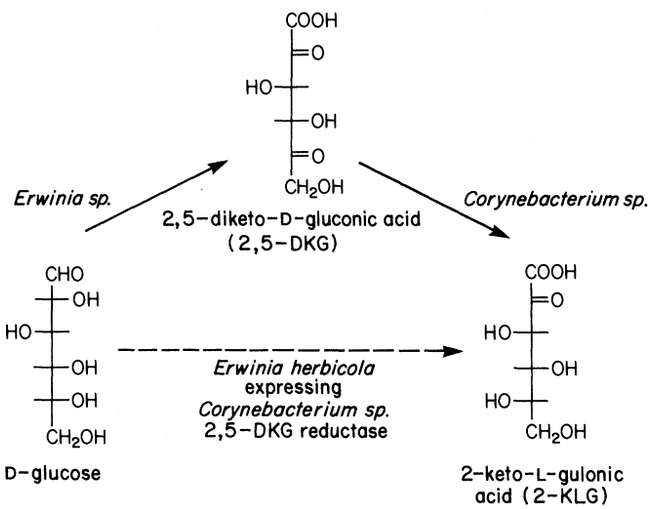

Fig. 2. Synthesis of 2-keto-L-gulonic acid by fermentation. The upper pathway shows the tandem fermentation route from glucose to 2-keto-L-gulonic acid via a 2,5-diketo-D-gluconic acid intermediate (*7*). The lower pathway shows the single fermentation that is made possible by combining the relevant traits of *Erwinia sp.* and *Corynebacterium sp.* in a single recombinant microorganism.

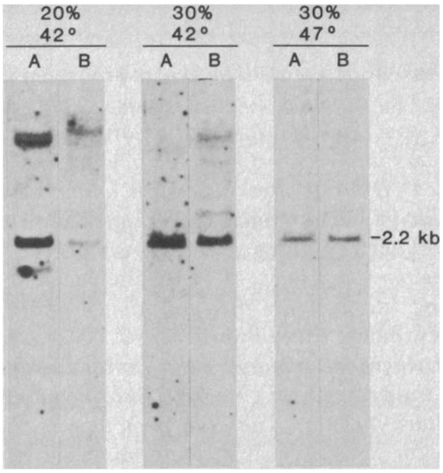

Fig. 3. Genomic Southern blots of *Corynebacterium sp.* DNA. The NH_2-terminal sequence of the purified 2,5-DKG reductase was used to design two long DNA probes (*12–14*). Probe A (CTCCATCCCGCAGCTGGGCTACGGCGTGTTCAAGGTGCCGCCG) had the same sense as the coding strand of the 2,5-DKG reductase gene and was derived from amino acids 11 to 25, probe B (GGCCTCCTCCACGGCGCGCTGGGCGTCGGCCGGCGGCACCTTG) had the sense of the complementary (noncoding) strand and was derived from amino acids 21 to 35 (Fig. 4). *Corynebacterium sp.* cells were lysed (*29*), and the DNA was purified by proteinase K treatment, phenol and chloroform extraction, and CsCl equilibrium density gradient centrifugation; the size of the purified DNA as assayed by agarose gel electrophoresis was approximately 100 kb. Bam HI digests of total DNA were fractionated by size on agarose gels and blotted onto nitrocellulose filter paper (*30*). Probes A and B were phosphorylated with [γ-^{32}P]ATP (adenosine triphosphate) and polynucleotide kinase and hybridized with the DNA on the filters in 50 m*M* NaH_2PO_4 (*p*H 7.0), 10 m*M* EDTA, 750 m*M* NaCl, 0.1 percent bovine serum albumin, 0.1 percent polyvinylpyrrolidone, 0.1 percent Ficoll, 0.1 percent sodium pyrophosphate, denatured salmon sperm DNA (50 μg/ml), and 20 or 30 percent formamide for 16 hours at 42°C. (Before hybridization the filters were treated with the above solution for 1 hour at 42°C.) Filters were washed at 42°C or 47°C three times in 3 m*M* sodium citrate (*p*H 7.0), 30 m*M* NaCl, 0.1 percent SDS, 0.1 percent sodium pyrophosphate for 30 minutes each time. Filters were blotted dry and autoradiographed with intensifying screens at −70°C. The percentage of formamide in the hybridization mix and the temperature of the wash were varied to find the set of conditions that yielded the best signal and the least amount of nonspecific hybridization for each probe. Shown in the figure are three sets of conditions for each probe. A procedure with the use of 30 percent formamide in the hybridization and a washing at 42°C (middle panel) was optimal and was used for subsequent colony hybridizations.

behavior of the *Corynebacterium sp.* DNA was examined in order to determine its base composition (*14*). The *Corynebacterium sp.* DNA was, in fact, found to be extremely GC-rich (71 percent G+C); therefore two 43-nucleotide long probes (Fig. 3) were designed from regions of the NH_2-terminal amino acid sequence with codons known to be prevalent in bacterial DNA's of high G+C content (*13*).

Under the appropriate conditions, the probes hybridized coincidentally to a 2.2-kb Bam HI fragment of *Corynebacterium sp.* DNA (Fig. 3). Therefore, a preparative Bam HI digest of *Corynebacterium sp.* DNA was fractionated by agarose gel electrophoresis, and DNA fragments in the size range 2.0 to 2.5 kb were isolated by electroelution and

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                                                                             MET THR VAL
   1 CCATGGACGGCAGCCCGAACGATCCCGACACCATCGTGCTCGACCCGCACGCCTGGAGGGCGCGGACCGCGCCTACCCTGGAAGAC ATG ACA GTT
     Nco I
                                         *** **  **  *** **  **  *** *** **  *** *** *** *** ***
     PRO SER ILE VAL LEU ASN ASP GLY ASN SER ILE PRO GLN LEU GLY TYR GLY VAL PHE LYS VAL PRO PRO ALA ASP
  96 CCC AGC ATC GTG CTC AAC GAC GGC AAT TCC ATT CCC CAG CTC GGG TAC GGC GTC TTC AAG GTG CCG CCG GCG GAC
                                                                           * *** *** *** *** **  ***
     THR GLN ARG ALA VAL GLU GLU ALA LEU GLU VAL GLY TYR ARG HIS ILE ASP THR ALA ALA ILE TYR GLY ASN GLU
 171 ACC CAG CGC GCC GTC GAG GAA GCG CTC GAA GTC GGC TAC CGG CAC ATC GAC ACC GCG GCG ATC TAC GGA AAC GAA
      ** *** *** *** **  *** **  **
     GLU GLY VAL GLY ALA ALA ILE ALA ALA SER GLY ILE ALA ARG ASP ASP LEU PHE ILE THR THR LYS LEU TRP ASN
 246 GAA GGC GTC GGC GCC GCG ATC GCG GCG AGC GGC ATC GCG CGC GAC GAC CTG TTC ATC ACG ACG AAG CTC TGG AAC

     ASP ARG HIS ASP GLY ASP GLU PRO ALA ALA ALA ILE ALA GLU SER LEU ALA LYS LEU ALA LEU ASP GLN VAL ASP
 321 GAT CGC CAC GAC GGC GAT GAG CCC GCT GCA GCG ATC GCC GAG AGC CTC GCG AAG CTG GCA CTC GAT CAG GTC GAC
                                         Pst I
     LEU TYR LEU VAL HIS TRP PRO THR PRO ALA ALA ASP ASN TYR VAL HIS ALA TRP GLU LYS MET ILE GLU LEU ARG
 396 CTG TAC CTC GTG CAC TGG CCG ACG CCC GCC GCC GAC AAC TAC GTG CAC GCG TGG GAG AAG ATG ATC GAG CTT CGC

     ALA ALA GLY LEU THR ARG SER ILE GLY VAL SER ASN HIS LEU VAL PRO HIS LEU GLU ARG ILE VAL ALA ALA THR
 471 GCA GCC GGT CTC ACC CGC AGC ATC GGC GTC TCG AAC CAC CTC GTG CCG CAC CTC GAG CGC ATC GTC GCC GCC ACC

     GLY VAL VAL PRO ALA VAL ASN GLN ILE GLU LEU HIS PRO ALA TYR GLN GLN ARG GLU ILE THR ASP TRP ALA ALA
 546 GGC GTC GTG CCG GCG GTG AAC CAG ATC GAG CTG CAC CCC GCC TAC CAG CAG CGC GAG ATC ACC GAC TGG GCC GCC

     ALA HIS ASP VAL LYS ILE GLU SER TRP GLY PRO LEU GLY GLN GLY LYS TYR ASP LEU PHE GLY ALA GLU PRO VAL
 621 GCC CAC GAC GTG AAG ATC GAA TCG TGG GGG CCG CTC GGT CAG GGC AAG TAC GAC CTC TTC GGC GCC GAG CCC GTC

     THR ALA ALA ALA ALA ALA HIS GLY LYS THR PRO ALA GLN ALA VAL LEU ARG TRP HIS LEU GLN LYS GLY PHE VAL
 696 ACT GCG GCT GCC GCC GCC CAC GGC AAG ACC CCG GCG CAG GCC GTG CTC CGT TGG CAC CTG CAG AAG GGT TTC GTG
                                                                             Pst I
     VAL PHE PRO LYS SER VAL ARG ARG GLU ARG LEU GLU GLU ASN LEU ASP VAL PHE ASP PHE ASP LEU THR ASP THR
 771 GTC TTC CCG AAG TCG GTC CGC CGC GAG CGC CTC GAA GAG AAC CTC GAC GTG TTC GAC TTC GAC CTC ACC GAC ACC

     GLU ILE ALA ALA ILE ASP ALA MET ASP PRO GLY ASP GLY SER GLY ARG VAL SER ALA HIS PRO ASP GLU VAL ASP
 846 GAG ATC GCC GCG ATC GAC GCG ATG GAT CCG GGC GAC GGT TCG GGT CGC GTG AGC GCA CAC CCC GAT GAG GTC GAC
         Δ270                         Bam HI              |→ URF
 921 TGA CCCCGCCGAACACCCGGAGGCCACGGGCGCAGGACTAGCCTGGGCTCGTGGCATCCCGGCTCTCCGAAGCGACCAGCCCCTACCTGCGCGCCCAC

1019 GCCGACAACCCCGTCGCATGGTGGCCGTGGGGCGAGGCGGCCTTCGCAGAAGCCCGTCGCCGCGACGTGCCCGTGATGGTCTCGATCGGCTACTCGACAT

1119 GCCACTGGTGCCACGTGATGGCACGGGAGAGCTTCGAGGATGCCGCGGTCGCCGCCGACCTCGACGCAGGGTTCGTCGCCGTCAAGGTCGACCGCGAGGA

1219 GCATCCCGAGGTCGACGCGGCCTACATGGCGGCCGCCGCCGCATTCACGCAGAACCTCGGCTGGCCGCTCACCGTCTTCGTCACACCCGCGGGGCGACCG

1319 TTCTTCGCGGGAACCTACTTCCCACCCGAACCGCGCGGCGGACTGCCTGCGTTCCGGCAGGTGCTCGCCGCCGTCGACGAGGCCTGGACCGAACGCCGCG

1419 ACCAGATCGAGAGCACAGGCGGCGCGATCGTGGATGCCCTCGCCGAGGTGCGGGGCGTCGCCGGTGCCGAGGCGTACGCGCTGCCGTCCGTCGACGACCT

1519 CGCCGCTGCCGCGACGGCGCTCGCCGCCCGTGAAGACACGGAGTTCGGCGGGTTCGGCGCAGCGGGCGGATCGCTCGAACAGCCCAAGTTCCCCGTCGCG

1619 ACCGCACTGCGGTTCCTGCAG
                    Pst I
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Fig. 4. Sequence of the *Corynebacterium sp.* 2,5-DKG reductase gene. Shown is a portion of the sequence of the cloned 2.2-kb Bam HI fragment starting from an Nco I site 86 nucleotides "upstream" from the 2,5-DKG reductase gene. The Bam HI site is at nucleotide 869, and the overlapping 0.9-kb Pst I fragment referred to in the text runs from nucleotide 753 to the end of the sequence. The region shown was sequenced by the dideoxy nucleotide chain termination method (*31*) from fragments subcloned in the vectors M13mp8 and M13mp9 (*32*). The 2,5-DKG reductase coding region and the 5′-untranslated region were sequenced on both strands. Because of its extremely high G+C content, the sequence of the entire region was also verified by resequencing with reaction mixtures containing deoxyinosine nucleoside triphosphate (*33*). The inverted repeat immediately preceding the start codon is indicated by converging arrows, and the Shine-Dalgarno sequence (*17*) is boxed. The amino acid sequences experimentally obtained from the NH_2-terminus and tryptic fragments of purified 2,5-DKG reductase are underscored. Asterisks indicate positions in which the sequences of the probes (Fig. 3) used to isolate the 2,5-DKG reductase clone match the gene sequence; each of the two 43-nucleotide probes was approximately 85 percent homologous to the corresponding gene sequence. The precise deletion (Δ270) of all DNA after the stop codon at position 921 was produced by site-directed in vitro mutagenesis on an M13 template with a deletion primer (*34*); the size and activity of 2,5-DKG reductase remained unchanged after the deletion (*24*). A long unassigned reading frame (URF) begins with a GTG codon at position 971 and continues to the Pst I site without interruption by a stop codon. This URF presumably represents a protein coding gene that immediately follows the 2,5-DKG reductase gene in an operon, but its identity is unknown.

cloned into the Bam HI site of pBR322. Colonies from the resultant partial genomic library were screened on duplicate filters under the appropriate conditions and clones containing the specifically hybridizing 2.2-kb Bam HI fragment were isolated. The DNA sequence of the 2.2-kb Bam HI fragment (Fig. 4) indicated that it did not contain the entire 2,5-DKG reductase gene. Hence, an overlapping 0.9-kb Pst I fragment, containing the coding region for the COOH-terminus of the 2,5-DKG reductase, was cloned with a 0.12-kb Pst I–Bam HI fragment (nucleotides 753 to 874 in Fig. 4) as a probe.

The sequence of the gene for 2,5-DKG reductase (Fig. 4) indicated that it coded for a 278-residue, acidic, alanine-rich protein (30,105 daltons). Because the high G+C content of the DNA (Fig. 4) made DNA sequencing difficult, precautions were taken to ensure that the reading frame was correctly assigned. The amino acid sequence predicted by the DNA sequence matched the NH_2-terminal protein sequence and several tryptic peptide sequences (Fig. 4) from the purified 2,5-DKG reductase. The first in-frame stop codon was the TGA at position 921 (Fig. 4). This was consistent with a deletion experiment in which the size and activity of the *Corynebacterium sp.* DNA 3′ to this stop codon (Fig. 4) was removed by primer-directed in vitro mutagenesis, and with carboxypeptidase B digestion of the purified 2,5-DKG reductase, which indicated that the COOH-terminal residue was aspartic acid (*9*).

Between the Nco I site (position 1) and the ATG start codon (position 87) there were no recognizable promoter sequences. Furthermore, insertion of lac, trp, or tac II promoters (*15*) "upstream" of the Nco I site produced no detectable expression in *Escherichia coli* strain MM294 (*16*). The only sequence resembling a Shine-Dalgarno sequence (*17*) was the GGAAG at position 80, and only two nucleotides separated this sequence from the ATG start codon. This small separation may in part explain the inefficient expression of the 2,5-DKG reductase gene in *E. coli* even when it was transcribed from a strong promoter (*18*). The putative Shine-Dalgarno sequence was also on the edge of a region of strong dyad symmetry (Fig. 4). If present in the messenger RNA (mRNA), this region could form a very stable (−29 kcal/mol) hairpin structure that would partially sequester the ribosome binding site and thereby inhibit translation of the message in *E. coli* (*19*). *Corynebacterium sp.* is a Gram-positive organism and similar stem-loop structures have been implicated in translational regulation of genes in other Gram-positive bacteria (*20*), hence expression of the 2,5-DKG reductase gene in *Corynebacterium sp.* may be modulated at the translational level.

Expression of the 2,5-DKG reductase gene. Expression of the 2,5-DKG reductase gene was accomplished by deleting the *Corynebacterium sp.* DNA upstream of the ATG start codon and inserting transcriptional and translational control sequences that work efficiently in *E. coli*. The *E. coli* trp promoter and a synthetic ribosome binding site from the plasmid pHGH207-1ptrpΔRI5′ (*15*) were attached to the 2,5-DKG reductase coding region (Fig. 5). Transformation of *E. coli* strain MM294 with the resultant plasmid, pmit12Δ/trp1, and selection for

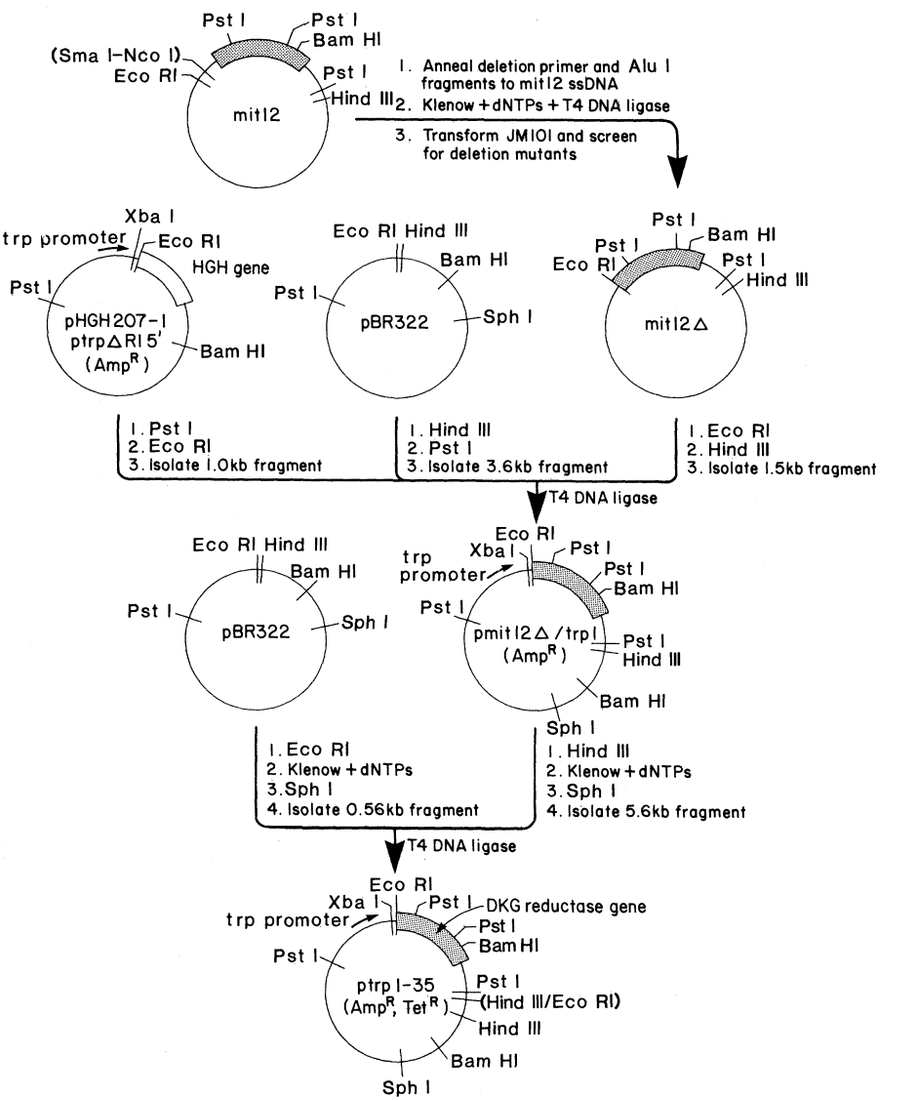

Fig. 5. Construction of the 2,5-DKG reductase expression vector. The 1.6-kb region of the *Corynebacterium sp.* genome shown in Fig. 4 was subcloned in M13mp9 cut by Sma I–Pst I (*32*) from the overlapping 2.2-kb Bam HI and 0.9-kb Pst I fragments described in the text. Single-stranded template from this subclone ("mit12") was annealed to a deletion primer (sequence: ACGGCCAGTGAATTCTATGACAGTTCCCAGC) and Alu I fragments of M13mp9 DNA, and this was treated with *E. coli* DNA polymerase I Klenow fragment, T4 DNA ligase, and deoxynucleoside triphosphates to synthesize heteroduplex mit12 RF molecules in vitro (*34*). These were used to transform *E. coli* strain JM101, and the resulting phage were screened by plaque hybridization to isolate recombinants containing the desired deletion. The effect of this deletion was the removal of the *Corynebacterium sp.* DNA between the Nco I site and the ATG start codon. Replicative form DNA was prepared from one such isolate (mit12Δ), and the 2,5-DKG reductase gene was excised by an Eco RI–Hind III digestion. This was ligated with the 1.0-kb Pst I–Eco RI fragment from the plasmid pHGH207-1ptrpΔRI5′ (*15*) and the 3.6-kb Pst I–Hind III fragment from pBR322. The resultant plasmid (pmit12Δ/trp1) was cut with Hind III, treated with *E. coli* DNA polymerase I Klenow fragment and deoxynucleoside triphosphates to create flush ends, and cut with Sph I; a 0.56-kb Eco RI (flush) Sph I fragment of pBR322, bearing the complete promoter for the tetracycline resistance gene, was then ligated to the above to create a 2,5-DKG reductase expression plasmid "ptrp1-35" that conferred tetracycline resistance in *E. coli*.

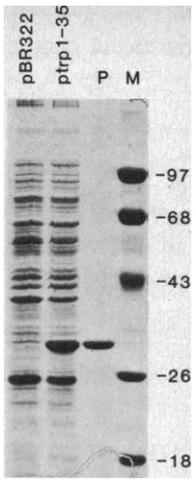

Fig. 6. Expression of 2,5-DKG reductase in *E. herbicola*. *Erwinia herbicola* cells transformed with ptrp1-35 or pBR322 were grown to saturation in LB broth containing tetracycline (5 μg/ml), and centrifuged; the sedimented cells were lysed with egg white lysozyme and Tween 80. Extracts were treated with deoxyribonuclease I to reduce viscosity, clarified by centrifugation, and assayed for 2,5-DKG reductase activity. Assays (1 ml) were performed in 150 m*M* tris-HCl (*p*H 7.5) buffer containing 200 μ*M* NADPH, 8.4 m*M* 2,5-DKG, and 100 μl of extract (see text). Activities were normalized to total protein content by means of absorbance measurements at 280 nm (A_{280}) on cell extracts. Extracts were also analyzed by electrophoresis on a 10 percent polyacrylamide–SDS gel (*35*). Lanes: pBR322, extract from pBR322-transformed cells; ptrp1-35, extract from ptrp1-35–transformed cells; P, purified *Corynebacterium sp.* 2,5-DKG reductase; M, protein size markers (molecular mass in kilodaltons) are shown in the margin.

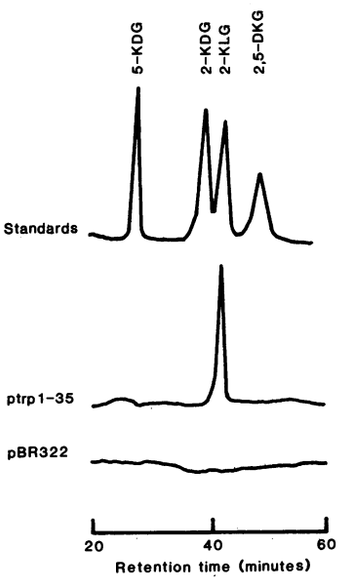

Fig. 7. Production of 2-KLG from glucose by ptrp1-35 transformed *E. herbicola*. *Erwinia herbicola* cells transformed with ptrp1-35 or pBR322 were grown to saturation in ATCC medium 1038 (*25*) containing tetracycline (5 μg/ml); they were harvested by centrifugation and resuspended in fresh 1038 medium containing tetracycline (5 μg/ml) and glycerol (20 mg/ml). Incubation of the cells was continued for 48 hours at 30°C, and the broths were then analyzed by ion-exchange HPLC (Aminex A-27 column, 250 mm by 4.6 mm; 1 ml/min with 0.2*M* ammonium formate, *p*H 3.2, at 45°C) (*36*). The elution stream was monitored by a refractive index detector. A single peak eluting with a retention time identical to a 2-KLG standard was observed for the ptrp1-35 broth; no such peak was observed for the pBR322 broth. 5-KDG, 5-keto-D-gluconic acid; 2-KDG, 2-keto-D-gluconic acid.

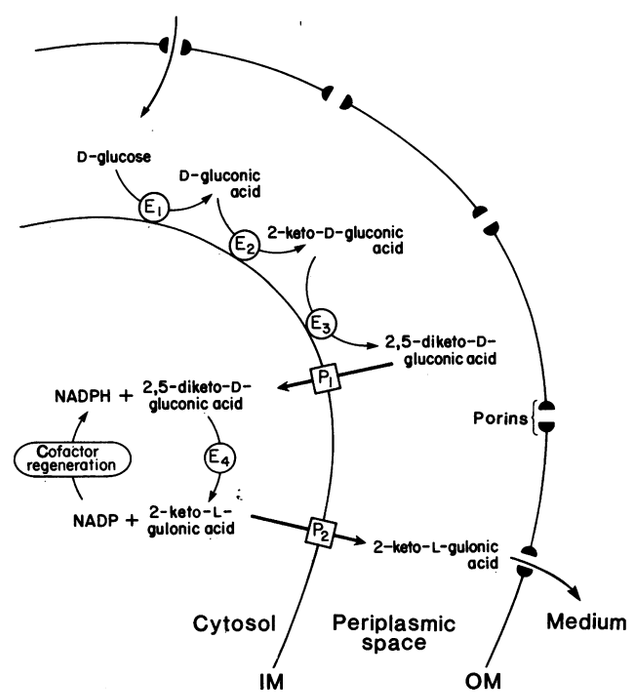

Fig. 8. Model for conversion of D-glucose to 2-KLG by recombinant *E. herbicola*. E_1, E_2, and E_3 represent membrane-bound, electron transport–linked dehydrogenases (*27*) that presumably convert D-glucose to 2,5-DKG in the periplasmic space. P_1 is a putative 2,5-DKG permease. E_4 is the cloned *Corynebacterium sp.* 2,5-DKG reductase. P_2 represents the efflux mechanism for 2-KLG, which may in fact be passive and nonspecific diffusion (*37*). IM and OM represent the *E. herbicola* inner and outer membranes, respectively. Also shown are the porins which regulate the diffusion of low molecular weight solutes through the outer membrane (*28*).

ampicillin-resistant transformants yielded a recombinant *E. coli* that expressed the 2,5-DKG reductase gene. Soluble extracts of whole cells exhibited NADPH-linked 2,5-DKG reductase activity, and fractionation of such extracts by SDS–polyacrylamide gel electrophoresis and subsequent immunoblotting (*21*) with rabbit antiserum to 2,5-DKG reductase and ^{125}I-labeled protein A showed a protein band with a mobility identical to that of purified *Corynebacterium sp.* 2,5-DKG reductase.

Because growth of *E. herbicola* (ATCC 21998) was found to be relatively insensitive to ampicillin (*22*), resistance to this antibiotic could not be used as a selectable marker in this organism. Therefore, to express the 2,5-DKG reductase gene in *E. herbicola*, pmit12Δ/trp1 was treated as described in Fig. 5 to reconstruct the promoter for the tetracycline resistance gene. The resultant plasmid, ptrp1-35, was used to transform *E. herbicola* (*23*), and tetracycline-resistant transformants were isolated. The 2,5-DKG reductase activity in the soluble cell extracts of ptrp1-35–transformed *E. herbicola* was 55.4 nmol/min per A_{280} unit compared to the activity from control cells transformed with pBR322, which was 1.2 nmol/min. When fractionated by SDS–polyacrylamide gel electrophoresis, the ptrp1-35 extracts exhibited a prominent band of the correct mobility for 2,5-DKG reductase that was not present in extracts from pBR322-transformed cells (Fig. 6). Immunoblots confirmed the identity of this band as 2,5-DKG reductase.

Surprisingly, expression of 2,5-DKG reductase was approximately 50 to 100 times higher in *E. herbicola* than in *E. coli* MM294 when the same expression plasmid (ptrp1-35) was used. Expression of human growth hormone with the use of the same control sequences (pHGH207-1ptrpΔRI5′) was only twice as high in *E. herbicola* compared to *E. coli*, and both expressed human growth hormone rather well (2 to 5 percent of total soluble protein). The reason for the extremely low expression levels of 2,5-DKG reductase in *E. coli* but not in *E. herbicola* is not clear at present. However, this property may be due to some intrinsic feature of the 2,5-DKG reductase gene sequence because deletion of downstream *Corynebacterium sp.* sequences (Fig. 5) produced no marked changes in the expression in either *E. coli* or *E. herbicola* (*24*). "Readthrough" expression of the tetracycline resistance gene from the trp promoter was also detected in *E. herbicola* but not in *E. coli* (*24*). This suggests that the difference in

expression is due to decreased transcription of the 2,5-DKG reductase gene in *E. coli* relative to *E. herbicola*, but differences in protein or mRNA stability cannot be ruled out.

Production of 2-keto-L-gulonic acid from D-glucose by ptrp1-35–transformed *E. herbicola*. *Erwinia herbicola* cells transformed with ptrp1-35 were grown to saturation in a D-glucose–containing medium (ATCC medium 1038) (*25*), then centrifuged and resuspended in fresh medium 1038 containing 2 percent glycerol. After further incubation the broth was analyzed by HPLC and found to contain 2-keto-L-gulonic acid at 1 g/liter but no detectable glucose or other organic acids (Fig. 7). The identity of the 2-keto-L-gulonic acid was confirmed by gas chromatography, mass spectrometry, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, optical rotation, and melting point analyses (*26*). A control culture, in which *E. herbicola* cells transformed with pBR322 were incubated in a similar manner, contained no 2-keto-L-gulonic acid (Fig. 7).

Conclusions. We have demonstrated here the usefulness of recombinant DNA technology for combining metabolic traits from two quite dissimilar microorganisms, *E. herbicola* and *Corynebacterium sp.*, in a single recombinant organism. This organism is capable of carrying out in a single fermentative step the bioconversion of D-glucose into 2-keto-L-gulonic acid, a valuable intermediate in the production of L-ascorbic acid.

In the present example separate biochemical pathways from the two organisms were joined by cloning and expressing a gene from one in the other. Some of the complexity inherent in even this simple case of metabolic pathway engineering is shown in Fig. 8. The dehydrogenases that serially oxidize D-glucose to 2,5-diketo-D-gluconic acid are membrane-bound periplasmic enzymes linked to the electron transport chain (*26, 27*) whereas the 2,5-diketo-D-gluconic acid reductase is soluble, NADPH-linked, and cytoplasmic. Therefore, an effective interface between these two topologically separate pathways requires transport of 2,5-diketo-D-gluconic acid across the inner cell membrane (Fig. 8), a step that may be mediated by a specific permease. In addition, efficient operation of the glucose to 2-keto-L-gulonic acid bioconversion requires an active NADPH regeneration system and some means of 2-keto-L-gulonic acid efflux from the cell. Exchange of metabolites between the periplasmic space and the medium, via diffusion through porin channels (*28*), is also a necessary component of this process (Fig. 8). The use of this or any similar scheme for the cost-effective production of L-ascorbic acid will require that all of these elements, not just the immediate enzymatic activities, be present and appropriately regulated.

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