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## Characterization of an L-Ascorbate Catabolic Pathway with Unprecedented Enzymatic Transformations

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### Abstract

L-Ascorbate (vitamin C) is ubiquitous in both our diet and the environment. Here we report that *Ralstonia eutropha* H16 (*Cupriavidus necator* ATCC 17699) uses L-ascorbate as sole carbon source via a novel catabolic pathway. RNAseq identified eight candidate catabolic genes, sequence similarity networks and genome neighborhood networks guided predictions for function of the encoded proteins, and the predictions were confirmed by *in vitro* assays and *in vivo* growth phenotypes of gene deletion mutants. L-Ascorbate, a lactone, is oxidized and ring-opened by enzymes in the cytochrome b<sub>561</sub> and gluconolactonase families, respectively, to form 2,3-diketo-L-gulonate. A protein predicted to have a WD40-like fold catalyzes an unprecedented benzilic acid rearrangement involving migration of a carboxylate group to form 2-carboxy-L-lyxonolactone; the lactone is hydrolyzed by a member of the amidohydrolase superfamily to yield 2-carboxy-L-lyxonate. A member of the PdxA family of oxidative decarboxylases catalyzes a novel decarboxylation that uses NAD<sup>+</sup> catalytically. The product, L-lyxonate, is catabolized to  $\alpha$ -ketoglutarate by a previously characterized pathway. The pathway is found in hundreds of bacteria, including the pathogens *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

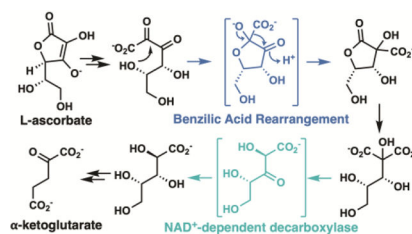
### Graphical Abstract

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**Supporting Information.** The Supporting Information is available free of charge on the ACS Publications website. Experimental details, RNA-Seq results, sequence similarity networks, genome neighborhood networks, NMR spectra, and mass spectra values (PDF)

List of organisms encoding L-ascorbate catabolic enzymes with UniProt IDs, list of upregulated genes by RNASeq (xlsx)



L-Ascorbate (vitamin C), ubiquitous in nature, is synthesized in plants and mammals.<sup>1,2</sup> Two similar bacterial catabolic pathways, first characterized in *Escherichia coli* K-12, are encoded by two gene clusters (designated *ula* and *sga*) that encode anaerobic (*ula*) and aerobic (*sga*) pathways for the catabolism of L-ascorbate or its oxidized, hydrolyzed product, 2,3-diketo-L-gulonate (DKG).<sup>3,4</sup>

We previously characterized pathways for tetronate catabolism by *Ralstonia eutropha* H16 (*Cupriavidus necator* ATCC 17699), a soil bacterium.<sup>5</sup> We observed that *R. eutropha* also can utilize L-ascorbate as sole carbon source although its genome does not encode orthologs of the Ula or Sga enzymes (Supplementary Table S1). Because plants are known to convert L-ascorbate to L-threonate,<sup>6</sup> we hypothesized that our previously reported L-threonate catabolic pathway in *R. eutropha*<sup>5</sup> could contribute to environmental L-ascorbate catabolism. However, mutants missing genes encoding the enzymes in L-threonate catabolism were able to grow with L-ascorbate (Supplementary Figure S1).

We now describe a new ubiquitous bacterial catabolic pathway for L-ascorbate. The pathway includes two novel enzymes: the first, not a member of a characterized family, likely catalyzes a benzilic acid rearrangement involving migration of a carboxylate group; the second is the first decarboxylase reported to use NAD<sup>+</sup> as a cofactor.

RNAseq was used to quantify the abundance of whole cell transcripts in *R. eutropha* cells grown with L-ascorbate, succinate, or D-fructose as carbon source (Supplemental Figure S2). With L-ascorbate, eleven genes were upregulated >50-fold compared to succinate and >200-fold compared to D-fructose (Supplemental Data). Eight of the encoded proteins could be identified as enzymes in a putative catabolic pathway; these were located in three distinct gene clusters that encode sequential segments of the pathway (Figure 1).

The EFI-EST tool (<http://efi.igb.illinois.edu/efi-est/>) was used to generate sequence similarity networks (SSNs) of enzymes encoded by the candidate genes (Supplementary Figures S3-5).<sup>7</sup> SSNs were refined to generate isofunctional clusters; these SSNs were used to generate genome neighborhood networks (GNNs) using the EFI-GNT tool (<http://efi.igb.illinois.edu/efi-gnt/>). The proximities of the putative pathway genes with each other (Figure 1) suggested their functional relationship (Supplementary Table S2 and Figure S6).

One gene cluster (Module 3) encodes orthologs of the characterized enzymes in *Pseudomonas aeruginosa* PAO1 in L-lyxonate catabolism (Supplementary Table S3).<sup>8</sup> In the *P. aeruginosa* pathway, an ortholog of H16\_B0217 (LyxD) catalyzes the dehydration of L-lyxonate to form 2-keto-3-deoxy-L-lyxonate (Kdl). The subsequent dehydration of Kdl is catalyzed by a member (KdlD) of the fumarylacetoacetate hydrolase superfamily (FAH) to

form  $\alpha$ -ketoglutarate semialdehyde (Kgs), which is oxidized by an ortholog of H16\_B0212 (KgsD) to form  $\alpha$ -ketoglutarate, an intermediate in the citric acid cycle (Figure 1C).<sup>8</sup> We previously reported that the KdlD from the FAH superfamily can be replaced by a member of the dihydrodipicolinate synthase (DHDPS) superfamily (e.g. UniProt ID A0NP47 from *Labrenzia aggregata* IAM 12614).<sup>8</sup> These homologous enzymes from *R. eutropha* were heterologously expressed and display the predicted *in vitro* activities.

We deleted the genes encoding Module 3 in independent *R. eutropha* strains; strains missing *lyxD* or *kdlD* were unable to grow with L-ascorbate (Figure 2); complemented strains recovered wild type growth (Supplementary Figure S7). Strains missing *kgsD* had reduced growth rates with L-ascorbate (Figure 2); growth was likely enabled by the activity of two similar enzymes (H16\_B0130, 61% ID/73% sim, or H16\_B0322, 47% ID/58% sim, to KgsD); complemented strains recovered wild type growth (Supplementary Figure S7).

Because *R. eutropha* converted L-lyxonate into  $\alpha$ -ketoglutarate, a central metabolic intermediate, and L-lyxonate catabolic genes are essential for L-ascorbate catabolism, we hypothesized that the remaining five enzymes (encoded by Modules 1 and 2) must catalyze the upstream oxidation, lactone hydrolysis, and decarboxylation reactions to convert L-ascorbate to L-lyxonate.

Members of the transmembrane cytochrome  $b_{561}$  family (PF03188) that contains H16\_A3010 (LaaO in Module 1) catalyze reduction of dehydro-L-ascorbate (DHA) to L-ascorbate.<sup>9</sup> We did not characterize LaaO but predict that it catalyzes the oxidation of L-ascorbate to form DHA, the substrate for H16\_A3012 (*vide infra*) (Figure 1A). Mutants lacking *laaO* grew with L-ascorbate with reduced growth rates compared to wild type (Figure 2); complemented strains grew like wild type (Supplementary Figure S7). Growth of the mutant strains with L-ascorbate was consistent with spontaneous/nonenzymatic oxidation of L-ascorbate in aerobic conditions.<sup>10</sup>

Although nonenzymatic hydrolysis of DHA is well-known,<sup>11</sup> we observed that H16\_A3012 (DhaL, PF08450 SGL gluconolactonase family in Module 1) catalyzed the hydrolysis of DHA to form DKG ( $k_{\text{cat}} = 8.0 \pm 0.6 \text{ s}^{-1}$ ,  $K_{\text{m}} = 490 \pm 70 \text{ }\mu\text{M}$ ,  $k_{\text{cat}}/K_{\text{m}} = 1.6 \pm 0.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , Supplementary Figures S8A and S8B). Deletion of *dhaL* reduced the growth rate and growth yield of *R. eutropha* during L-ascorbate growth (Figure 2). The complemented strains grew like wild type with each carbon source (Supplementary Figure S7).

H16\_B1217 in Module 2 is predicted to have a WD40/YVTN repeat-like-containing domain (member of IPR015943). Proteins with these domains have been reported to catalyze various unrelated reactions, providing little predictive information about the function of H16\_B1217.<sup>12–14</sup> Incubation of H16\_B1217 with DKG leads to the production of 2-carboxy-L-lyxonolactone (Figure 1B) ( $k_{\text{cat}} = 0.6 \pm 0.1 \text{ s}^{-1}$ ,  $K_{\text{m}} = 81 \pm 7 \text{ }\mu\text{M}$ ,  $k_{\text{cat}}/K_{\text{m}} = 7.9 \pm 1.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ , Supplementary Figure S8C). Its identity is supported by LC-MS and <sup>1</sup>H and <sup>13</sup>C NMR spectroscopies as well as the observation that it is labile to hydrolysis. Labeling studies confirm the rearrangement (*vide infra*). The structure of 2-carboxy-L-lyxonolactone (Clx lactone) suggests that H16\_B1217 (Dkg mutase or DkgM) directs addition of the C5 hydroxyl group to the C2 carbonyl.

Two mechanisms can be envisioned for the subsequent rearrangement that generates the lactone: migration of the C1 carboxylate group from C2 to C3 via a concerted benzylic acid rearrangement or stepwise decarboxylation/carboxylation (transcarboxylation) involving CO<sub>2</sub> and a vicinal enediol as intermediate (Supplementary Figure S9). The nonenzymatic rearrangement of DKG to 2-carboxy-L-lyxonate (Clx, 2-carboxy-L-*threo*-pentonate) in alkaline conditions has been reported; the mechanism was assumed to be the benzylic acid rearrangement.<sup>15</sup>

To provide possible evidence to distinguish between the mechanisms, we incubated Clx with [<sup>13</sup>C]-bicarbonate at alkaline pH and also with DkgM. The nonenzymatic incubation produced no observable <sup>13</sup>C incorporation in the product ( $6.1 \pm 1.1\%$  <sup>13</sup>C-labeled Clx compared to  $5.3 \pm 1.4\%$  in nonlabelled control reactions Supplementary Figure S10), providing strong support for the benzylic acid rearrangement mechanism. The incubation with DkgM also does not lead to <sup>13</sup>C incorporation ( $5.5 \pm 2.3\%$  <sup>13</sup>C-labeled Clx compared to  $4.8 \pm 1.0\%$  in nonlabelled control reactions Supplementary Figure S11). However, the absence of <sup>13</sup>C incorporation in the DkgM-catalyzed reaction is consistent with either mechanism, if the CO<sub>2</sub> intermediate is sequestered in the active site. We prefer the benzylic acid rearrangement mechanism given the nonenzymatic counterpart.

L-Ascorbate-dependent growth of mutant strains lacking *dkgM* was abolished (Figure 2); complemented strains grew like wild type (Supplementary Figure S7).

We observed that H16\_B1219 (PF04909 amidohydrolase family; Clx lactone lactonase, ClxL in Module 2) catalyzes the hydrolysis of Clx lactone to yield Clx, ( $k_{\text{cat}} = 90 \pm 10 \text{ s}^{-1}$ ,  $K_{\text{m}} = 850 \pm 120 \text{ }\mu\text{M}$ ,  $k_{\text{cat}}/K_{\text{m}} = 1.0 \pm 0.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , Supplementary Figure S8D). Clx is a geminal dicarboxylate with carboxyl groups derived from C1 and C2 of DKG.

Incubation of Clx and NAD<sup>+</sup> with H16\_B0216 (PF04166 PdxA oxidative decarboxylase family in Module 2) generates L-lyxonate (Figure 1C), even with sub-stoichiometric amounts of NAD<sup>+</sup> ( $k_{\text{cat}} = 0.33 \pm 0.08 \text{ s}^{-1}$ ,  $K_{\text{m}} = 800 \pm 400 \text{ }\mu\text{M}$ ,  $k_{\text{cat}}/K_{\text{m}} = 4.0 \pm 2.0 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ , see Supplementary Figures S8E and S12). Characterized members of the PdxA family perform oxidative decarboxylations of  $\beta$ -hydroxy acids.<sup>5</sup> We propose that H16\_B0216 (Clx decarboxylase, ClxD) catalyzes the NAD<sup>+</sup>-dependent oxidation of the C3 alcohol to a ketone and NADH followed by a single decarboxylation of the  $\beta$ -keto diacid intermediate. ClxD then catalyzes reduction of the  $\beta$ -keto monoacid by the tightly bound NADH to yield L-lyxonate and regenerate the NAD<sup>+</sup> cofactor (Scheme 1).

A stopped-flow experiment with equivalent amounts of enzyme and substrate revealed a small, substoichiometric transient increase in absorbance at 340 nm (Supplementary Figure S13). This result may be explained by rate-limiting oxidation of the substrate to form the  $\beta$ -keto diacid intermediate and NADH followed by rapid decarboxylation and reduction of the resulting  $\beta$ -keto monoacid intermediate.

Tightly bound NAD<sup>+</sup> participates in other overall redox neutral enzymatic reactions,<sup>16</sup> but ClxD represents, to our knowledge, the first decarboxylase that uses NAD<sup>+</sup> as a cofactor.

Deletion of *clxD* abolished growth of *R. eutropha* with L-ascorbate (Figure 2); complemented strains recovered wild type growth with L-ascorbate (Supplementary Figure S7). The knockout of the orthologous gene in *P. aeruginosa* PAO1 (locus ID PA 2212, UniProt ID Q9IIQ5) does not affect growth with L-lyxonate.<sup>8</sup>

We used <sup>13</sup>C-labeled L-ascorbate to confirm the reaction sequence from L-ascorbate to L-lyxonate. Clx displays two resonances in its <sup>13</sup>C NMR spectrum at  $\delta$  177.4 and 177.7 ppm (diastereotopic). Starting with 1-[<sup>13</sup>C]-L-ascorbate, the  $\delta$  177.7 ppm resonance is enriched; starting with 2-[<sup>13</sup>C]-L-ascorbate, the  $\delta$  177.4 ppm resonance is enriched (Figure 3). Therefore, 1-[<sup>13</sup>C]-L-ascorbate produced 1-[<sup>13</sup>C]-L-lyxonate and CO<sub>2</sub>, and 2-[<sup>13</sup>C]-L-ascorbate produced L-lyxonate and <sup>13</sup>CO<sub>2</sub> (Supplementary Figures S14-16). This labeling requires that a bond is formed between C1 and C3 of the DKG substrate and the bond between C1 and C2 is cleaved to release CO<sub>2</sub> in the formation of the Clx lactone product (Figure 4).

As described earlier, the L-lyxonate product of the ClxD reaction is catabolized by the enzymes in module 3 (the *P. aeruginosa* pathway) to produce  $\alpha$ -ketoglutarate (Figure 1C).

The complete catabolic pathway from L-ascorbate to  $\alpha$ -ketoglutarate is encoded in the *R. eutropha* genome in three distinct neighborhoods, each encoding enzymes in one of the three functionally discrete modules (Figure 1). Albeit via a combination of enzymes from other families, Module 1 (from L-ascorbate to DKG) is shared with organisms that catabolize DKG using the *E. coli* catabolic pathways.<sup>3</sup> In the *E. coli* pathways, DKG is reduced, phosphorylated and decarboxylated to form D-xylulose 5-phosphate; in the *R. eutropha* pathway DKG is decarboxylated to form L-lyxonate.

DKG, the substrate for the second module in the *R. eutropha* pathway, is a spontaneous environmental degradation product of L-ascorbate<sup>10</sup> and is a likely catabolic starting point for the 148 organisms that have only the last two modules as determined by a combined SSN/GNN analysis (Supplemental Data).

2-Carboxy-L-lyxonate, a naturally occurring plant metabolite derived from L-ascorbate,<sup>10,17</sup> is a probable catabolic starting point for the 415 organisms that have only the third module (Supplemental Data).

In summary, we have reported identification, characterization, and annotation of the genes and enzymes involved in a previously unknown L-ascorbate catabolic pathway (Table 1, Figure S17) found in *R. eutropha*, and hundreds of other organisms, including the bacterial pathogens *P. aeruginosa* and *Acinetobacter baumannii* (Supplemental Data). The pathway includes two unprecedented enzymatic reactions: the first enzyme-catalyzed benzilic acid rearrangement and the first decarboxylase to use NAD<sup>+</sup> as a cofactor, not a cosubstrate.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGMENT

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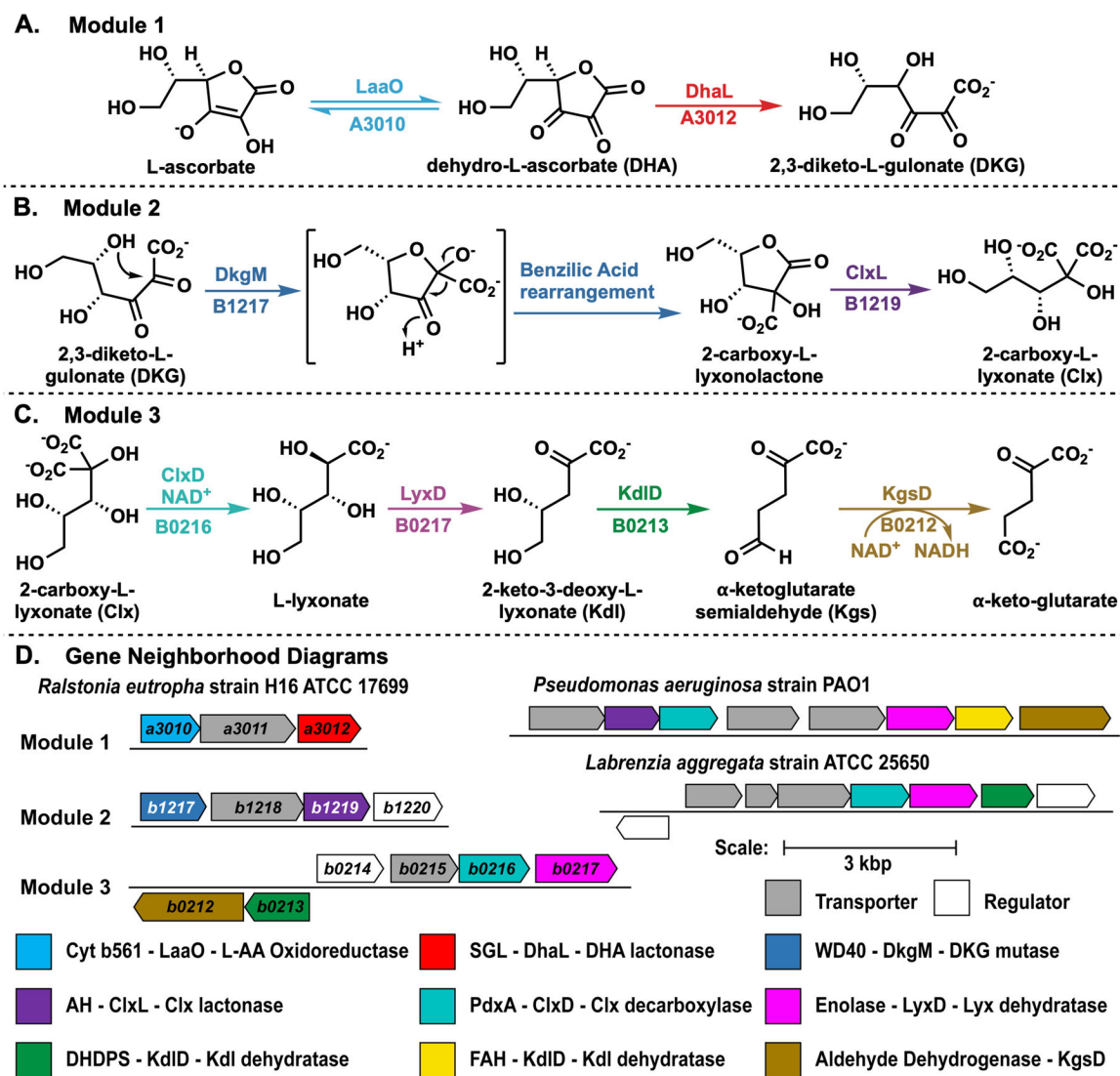
### Funding Sources

No competing financial interests have been declared.

## REFERENCES

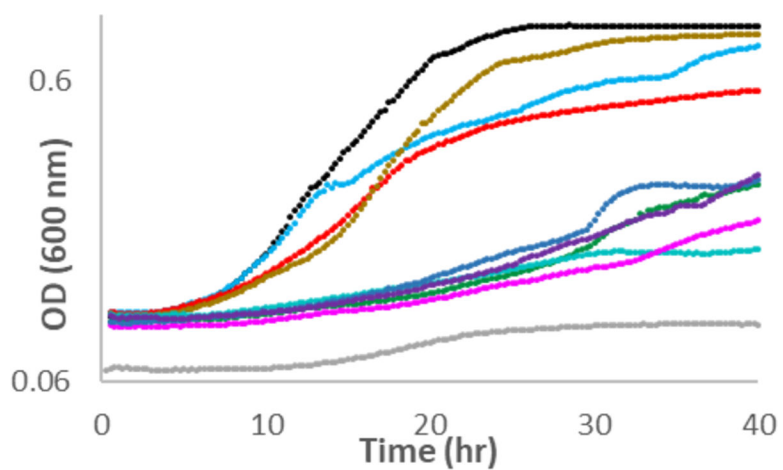
- (1). Hancock RD; Viola R Biosynthesis and Catabolism of L-Ascorbic Acid in Plants. *Critical Reviews in Plant Sciences* 2005, 24 (3), 167–188. 10.1080/07352680591002165.
- (2). Smirnoff N Ascorbic Acid Metabolism and Functions: A Comparison of Plants and Mammals. *Free Radic. Biol. Med* 2018, 122, 116–129. 10.1016/j.freeradbiomed.2018.03.033. [PubMed: 29567393]
- (3). Yew WS; Gerlt JA Utilization of L-Ascorbate by Escherichia Coli K-12: Assignments of Functions to Products of the Yjf-Sga and Yia-Sgb Operons. *J. Bacteriol* 2002, 184 (1), 302–306. 10.1128/JB.184.1.302-306.2002. [PubMed: 11741871]
- (4). Zhang Z; Aboulwafa M; Smith MH; Milton H Saier, J. The Ascorbate Transporter of Escherichia Coli. *Journal of Bacteriology* 2003, 185 (7), 2243–2250. 10.1128/JB.185.7.2243-2250.2003. [PubMed: 12644495]
- (5). Zhang X; Carter MS; Vetting MW; San Francisco B; Zhao S; Al-Obaidi NF; Solbiati JO; Thiaville JJ; de Crécy-Lagard V; Jacobson MP; Almo SC; Gerlt JA Assignment of Function to a Domain of Unknown Function: DUF1537 Is a New Kinase Family in Catabolic Pathways for Acid Sugars. *Proc. Natl. Acad. Sci. U.S.A* 2016, 113 (29), E4161–4169. 10.1073/pnas.1605546113. [PubMed: 27402745]
- (6). Truffault V; Fry SC; Stevens RG; Gautier H Ascorbate Degradation in Tomato Leads to Accumulation of Oxalate, Threonate and Oxalyl Threonate. *The Plant Journal* 2017, 89 (5), 996–1008. 10.1111/tpj.13439. [PubMed: 27888536]
- (7). Gerlt JA; Bouvier JT; Davidson DB; Imker HJ; Sadkhin B; Slater DR; Whalen KL Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST): A Web Tool for Generating Protein Sequence Similarity Networks. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 2015, 1854 (8), 1019–1037. 10.1016/j.bbapap.2015.04.015. [PubMed: 25900361]
- (8). Ghasempur S; Eswaramoorthy S; Hillerich BS; Seidel RD; Swaminathan S; Almo SC; Gerlt JA Discovery of a Novel L-Lyxonate Degradation Pathway in Pseudomonas Aeruginosa PAO1. *Biochemistry* 2014, 53 (20), 3357–3366. 10.1021/bi5004298. [PubMed: 24831290]
- (9). Lu P; Ma D; Yan C; Gong X; Du M; Shi Y Structure and Mechanism of a Eukaryotic Transmembrane Ascorbate-Dependent Oxidoreductase. *PNAS* 2014, 111 (5), 1813–1818. 10.1073/pnas.1323931111. [PubMed: 24449903]
- (10). Green MA; Fry SC Vitamin C Degradation in Plant Cells via Enzymatic Hydrolysis of 4-Oxalyl-L-Threonate. *Nature* 2005, 433 (7021), 83–87. 10.1038/nature03172. [PubMed: 15608627]
- (11). Deutsch JC Spontaneous Hydrolysis and Dehydration of Dehydroascorbic Acid in Aqueous Solution. *Analytical Biochemistry* 1998, 260 (2), 223–229. 10.1006/abio.1998.2700. [PubMed: 9657882]
- (12). Baker SC; Saunders NFW; Willis AC; Ferguson SJ; Hajdu J; Fülöp V Cytochrome C<sub>d1</sub> Structure: Unusual Haem Environments in a Nitrite Reductase and Analysis of Factors Contributing to  $\beta$ -Propeller Folds Edited by K. Nagai. *Journal of Molecular Biology* 1997, 269 (3), 440–455. 10.1006/jmbi.1997.1070. [PubMed: 9199411]

- (13). Plata G; Fuhrer T; Hsiao T-L; Sauer U; Vitkup D Global Probabilistic Annotation of Metabolic Networks Enables Enzyme Discovery. *Nature Chemical Biology* 2012, 8 (10), 848–854. 10.1038/nchembio.1063. [PubMed: 22960854]
- (14). Lin Z; Su X; Chen W; Ci B; Zhang S; Lin H Dph7 Catalyzes a Previously Unknown Demethylation Step in Diphthamide Biosynthesis. *J. Am. Chem. Soc* 2014, 136 (17), 6179–6182. 10.1021/ja5009272. [PubMed: 24739148]
- (15). Löwendahl L; Petersson G Conversion of Dehydroascorbic Acid to a Branched Hexaric Acid in Neutral and Alkaline Aqueous Solution. *Analytical Biochemistry* 1976, 72 (1), 623–628. 10.1016/0003-2697(76)90575-3. [PubMed: 7973]
- (16). Oppenheimer NJ NAD<sup>+</sup> and NADP<sup>+</sup> as Prosthetic Groups for Enzymes In *Encyclopedia of Life Sciences (ELS)*; John Wiley & Sons, 2010 10.1002/9780470015902.a0000637.pub2.
- (17). Parsons HT; Yasmin T; Fry SC Alternative Pathways of Dehydroascorbic Acid Degradation in Vitro and in Plant Cell Cultures: Novel Insights into Vitamin C Catabolism. *Biochemical Journal* 2011, 440 (3), 375–385. 10.1042/BJ20110939. [PubMed: 21846329]

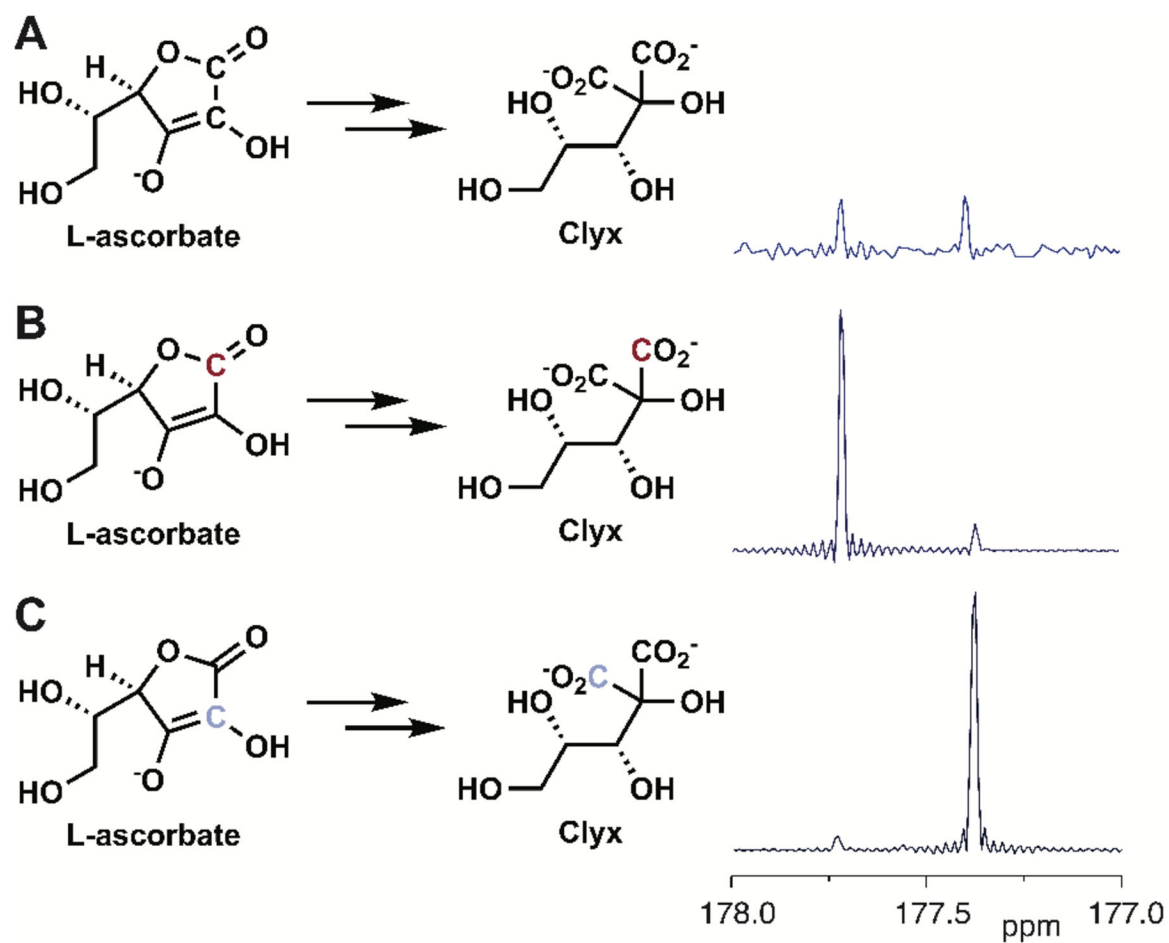


**Figure 1.** Catabolic pathway of L-ascorbate in *R. eutropha* occurs in three modules (A-C) that are encoded in distinct gene clusters (D). A) Module 1: L-Ascorbate is oxidized and hydrolyzed to 2,3-diketo-L-gulonate. B) Module 2: 2,3-diketo-L-gulonate is substrate for a benzilic acid rearrangement to yield 2-carboxy-L-lyxonolactone, which is ring-opened to 2-carboxy-L-lyxonate. C) Module 3: 2-carboxy-L-lyxonate is decarboxylated via oxidation and reduction of C3 to produce L-lyxonate, which is dehydrated twice and oxidized to generate  $\alpha$ -ketoglutarate, a citric acid cycle intermediate. D) Gene neighborhood diagrams mentioned in the main text.

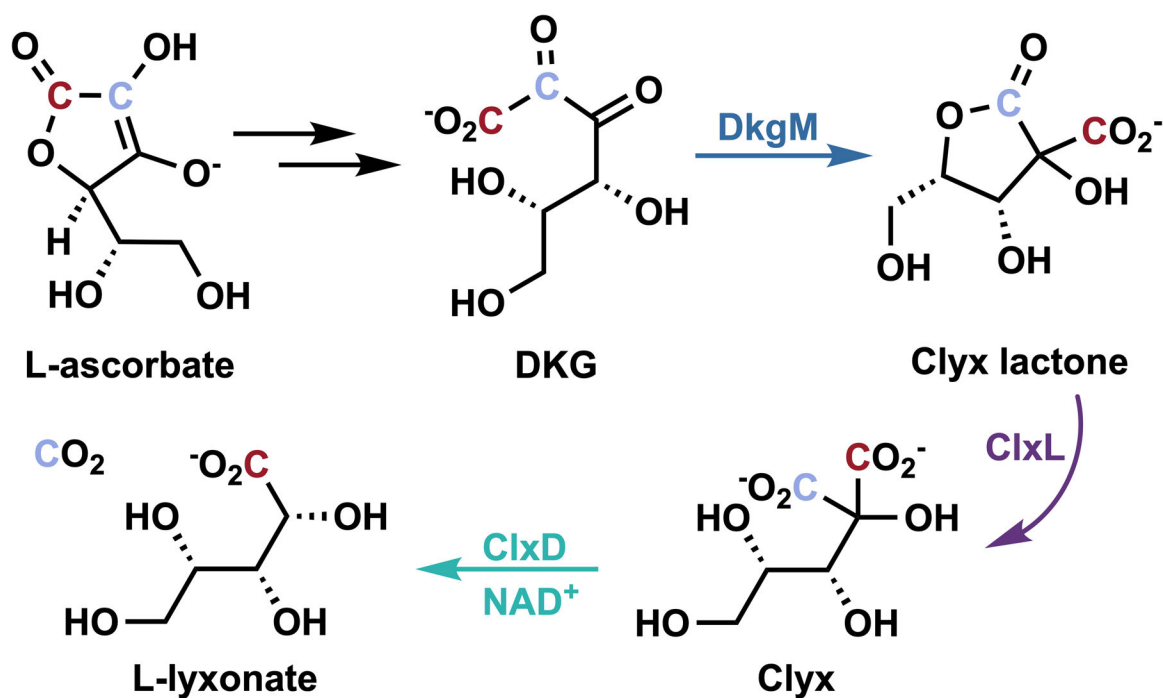




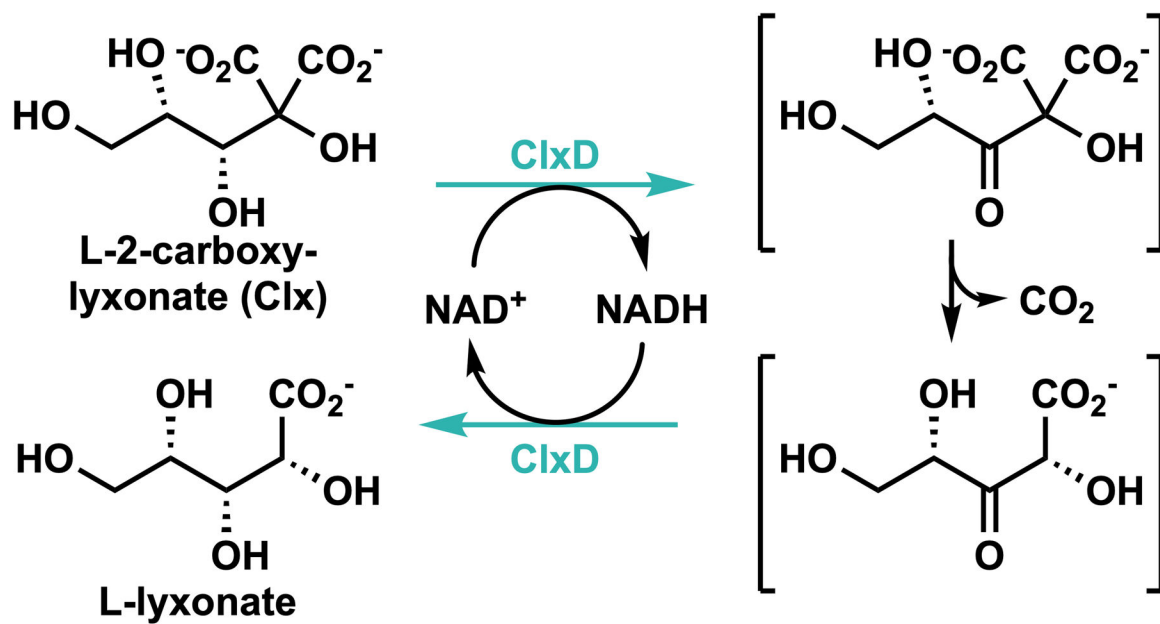
**Figure 2.** Growth of wild type (black) and mutant strains (lacking individual genes, correspondingly colored as in Figure 1) of *R. eutropha* with L-ascorbate as the sole carbon source.



**Figure 3.**  $^{13}\text{C}$  NMR Spectra of 2-carboxy-L-lyxonates. A) From unlabeled L-ascorbate, B) from 1- $^{13}\text{C}$ -L-ascorbate, and C) from 2- $^{13}\text{C}$ -L-ascorbate.



**Figure 4.** <sup>13</sup>C-labeled ascorbate was used to support our proposed pathway. The C1 of L-ascorbate becomes C1 of L-lyxonate, while C2 of L-ascorbate is removed as CO<sub>2</sub>.



**Scheme 1.**  
Proposed mechanism of the ClxD-catalyzed reaction

**Table 1.**

## UniProt IDs and L-Ascorbate Catabolic Pathway Enzyme Names

UniProt ID	Enzyme Name and Description
Q0K7D6	LaaO, L-ascorbate oxidoreductase
Q0K7D4	DhaL, DHA lactonase
Q0K1W7	DkgM, DKG mutase
Q0K1W5	ClxL, Clyx lactonase
Q0K4Q7	ClxD, Clx decarboxylase
Q0K4Q6	LyxD, L-lyxonate dehydratase
Q0K4R0	KdlD, Kdl dehydratase
Q0K4R1	KgsD, Kgs dehydrogenase

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