Functional Annotation Report: *leuD* (Q88LE7, PP_1986) in *Pseudomonas putida* KT2440

Gene/Protein Identity Verification

Before presenting the research, the target identity was verified against all mandatory checkpoints, and **no ambiguity was found**:

Checkpoint	Result
Gene symbol vs. protein description	**Consistent.** "leuD" is the universal bacterial symbol for the 3-isopropylmalate dehydratase **small subunit** / isopropylmalate isomerase (IPMI).
Organism	**Confirmed.** UniProt Q88LE7 and KEGG `ppu:PP_1986` both map to *Pseudomonas putida* KT2440 (ATCC 47054 / DSM 6125).
Family / domains	**Confirmed.** LeuD type-1 subfamily; IPMI_Swivel (IPR033940), aconitase small-subunit/swivel fold (IPR000573, IPR015928); Pfam Aconitase_C, LeuD, LeuD_C.
Literature vs. target	**Consistent.** All retrieved primary literature concerns the *leuC/leuD* isopropylmalate isomerase of leucine biosynthesis — the same protein. No conflicting "same-symbol, different-gene" literature was found.
Sequence orthology	**Confirmed.** 67.3 % amino-acid identity to biochemically characterized *E. coli* LeuD (see §6).

The gene symbol is unambiguous and the literature is directly relevant. The annotation below rests on experimental biochemistry in *Salmonella*/*E. coli*, X-ray crystallography in *Mycobacterium tuberculosis*, iron–sulfur biochemistry in yeast, and curated database records (UniProt/HAMAP, KEGG, Rhea), transferred to the *P. putida* protein through high-confidence orthology.

1. Summary (Answer to the Research Question)

leuD (Q88LE7, locus PP_1986) encodes the **small subunit of isopropylmalate isomerase (IPMI; 3-isopropylmalate dehydratase; EC 4.2.1.33)**, a 214-residue polypeptide. It has **no independent catalytic activity**; instead it forms an **obligate 1:1 heterodimer with the large subunit LeuC (PP_1985)**. The LeuCD holoenzyme catalyzes the **reversible isomerization of (2S)-2-isopropylmalate to (2R,3S)-3-isopropylmalate**, proceeding through the dehydration intermediate **2-isopropylmaleate**. This is the **second of four dedicated steps of L-leucine biosynthesis** (from 2-oxoisovalerate). The enzyme is a member of the **aconitase superfamily** of [4Fe-4S] hydro-lyases; the catalytic iron–sulfur cluster resides on LeuC, while **LeuD contributes the aconitase "swivel" domain and the substrate-binding/substrate-discriminating loops** that complete the active-site cleft. The reaction occurs in the **cytoplasm**. LeuD's role is therefore that of a **structural/substrate-specificity subunit essential for isomerase activity**, not a cofactor-bearing catalytic subunit.

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2. Primary Function: the Reaction Catalyzed and Substrate Specificity

Reaction (LeuCD holoenzyme):

> (2S)-2-isopropylmalate ⇌ (2R,3S)-3-isopropylmalate, via 2-isopropylmaleate (EC 4.2.1.33; Rhea RHEA:32287; ChEBI 35121 ⇌ 1178) [UniProt Q88LE7 / HAMAP MF_01031; KEGG K01704].

Mechanistically this is a **dehydration followed by re-hydration in the opposite orientation** — a net isomerization achieved by a hydro-lyase, exactly analogous to aconitase's citrate ⇌ isocitrate interconversion via *cis*-aconitate. UniProt annotates the function as *"Catalyzes the isomerization between 2-isopropylmalate and 3-isopropylmalate, via the formation of 2-isopropylmaleate"* [Q88LE7, HAMAP-Rule MF_01031].

Experimental support for the reaction and complex comes from classical work on the enterobacterial enzyme: - IPMI *"is a complex enzyme composed of two subunits which are coded for by two genes of the leucine operon, leuC and leuD … The native isopropylmalate isomerase was shown to have a Km for its substrate alpha-isopropylmalate of 3 × 10^{-4} M"* (Fultz & Kemper,

J. Bacteriol. 1981,).

P 7026530

- The functional complex *"catalyzes the stereospecific conversion reaction of α-isopropylmalate to β-isopropylmalate"* (Manikandan et al., 2011,).

P 20938981

Substrate specificity. The primary physiological substrate is **2-isopropylmalate** (the isopropyl-substituted malate). KEGG maps ortholog K01704 to a **secondary activity, EC 4.2.1.35 ((R)-2-methylmalate/citramalate dehydratase**, C5-branched dibasic acid metabolism, pathway ppu00660), reflecting the modest substrate promiscuity typical of aconitase-superfamily [4Fe-4S] hydro-lyases toward the closely related 2-methylmalate/citraconate. In *Leptospira interrogans*, the leucine-pathway IPMI (EC 4.2.1.33) participates in a threonine-independent isoleucine route acting on citraconate-type substrates, directly illustrating this family flexibility (Xu et al., 2004,).

P 15292141

The **superfamily assignment is explicit**: *"In the aconitase superfamily, which includes the archetypical aconitase, homoaconitase, and isopropylmalate isomerase..."* (Watanabe et al., 2016,).

P 27929065

3. Molecular Role of LeuD within the Enzyme

IPMI is an **obligate heterodimer**; LeuD alone is inert. Biochemical purification from *Salmonella typhimurium* resolved two copurifying polypeptides — **51 kDa (LeuC)** and **23.5 kDa (LeuD)** — and both are required, as shown by in-vitro complementation of *leuC* and *leuD* mutant extracts (Fultz & Kemper 1981,).

P 7026530

Genetic work confirmed that *"the isopropylmalate isomerase of Salmonella typhimurium and Escherichia coli is a complex of the leuC and leuD gene products"* (Stover et al., 1988,),

P 2838459

and that a 22-kDa LeuD functional analog (newD) can restore leucine prototrophy by pairing with LeuC (Kemper 1974,).

P 4612005

Division of labor: - **LeuC (large subunit)** harbors the catalytic **[4Fe-4S] cluster** and most active-site residues (aconitase domains 1–3). - **LeuD (small subunit, this protein)** supplies **domain 4, the "swivel" domain** of the aconitase fold. In single-chain aconitase this domain is part of one polypeptide; in bacterial IPMI the fold is **split across two genes**, and LeuD reconstitutes the active-site cleft at the LeuC–LeuD interface.

Crystallography of *M. tuberculosis* LeuD (to 1.2 Å) localized LeuD's two most flexible, functionally critical regions: *"the regions of residues 30-37, the substrate discriminating loop, and of residues 70-74, the substrate binding loop"* (Manikandan et al., 2011,).

P 20938981

The same study found *"the presence of two LeuD subfamilies"* — consistent with the UniProt assignment of Q88LE7 to **LeuD type-1** — and showed by solution scattering that the LeuC and LeuCD shapes differ radically from mitochondrial aconitase, underscoring the distinct two-chain architecture. Thus LeuD acts as a **structural + substrate-specificity subunit**: it completes the catalytic machinery and its loops discriminate the isopropylmalate substrate, but it does not itself carry the cofactor.

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4. Cofactor and Catalytic Chemistry

IPMI is a **[4Fe-4S] iron–sulfur enzyme**. The cluster (on LeuC) coordinates a substrate hydroxyl/carboxylate and activates it for dehydration, exactly as in aconitase. Evidence that catalytic competence depends on Fe–S assembly: - Apo-IPMI is activated by cluster transfer: *"The assembled Fe/S cluster could be transferred from SufU to the apo form of isopropylmalate isomerase Leu1, rapidly forming catalytically active [4Fe-4S]-containing holo-enzyme"* (Albrecht et al., 2010,).

P 20097860

- Impaired Fe–S biogenesis lowers IPMI activity, and IPMI is classed among *"cytosolic Fe-S enzymes (sulfite reductase and isopropylmalate isomerase)"* (Patil et al., 2012,).

P 23192348

LeuD itself does not bind the cluster but is required to build the catalytic site around it.

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5. Localization

Leucine biosynthesis in bacteria is a **cytoplasmic** process, and IPMI is a soluble cytoplasmic enzyme. In eukaryotes the orthologous isopropylmalate isomerase (Leu1) is explicitly described as a **cytosolic Fe-S enzyme** (Patil et al., 2012, P 23192348); in the prokaryote *P. putida*, which lacks such compartmentalization, the LeuCD complex operates in the cytoplasm. Consistent with this, UniProt Q88LE7 carries **no membrane, signal-peptide, or secretion annotation** — the protein is a soluble cytoplasmic subunit.

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6. Biological Pathway and Genomic Context in *P. putida*

Pathway placement. LeuCD catalyzes **step 2 of 4** of *"L-leucine from 3-methyl-2-oxobutanoate (2-oxoisovalerate)"* (UniProt PATHWAY; KEGG module **M00432**, "Leucine biosynthesis, 2-oxoisovalerate ⇒ 2-oxoisocaproate"):

1. **LeuA** (2-isopropylmalate synthase) — condenses 2-oxoisovalerate + acetyl-CoA → 2-isopropylmalate
2. **LeuCD** (this enzyme) — 2-isopropylmalate ⇌ 3-isopropylmalate (via 2-isopropylmaleate)
3. **LeuB** (3-isopropylmalate dehydrogenase, EC 1.1.1.85) — oxidative decarboxylation → 2-oxoisocaproate
4. **IlvE/aminotransferase** — transamination → **L-leucine**

KEGG also maps PP_1986 to valine/leucine/isoleucine biosynthesis (ppu00290), 2-oxocarboxylic-acid metabolism (ppu01210), C5-branched dibasic acid metabolism (ppu00660), and the biosynthesis-of-amino-acids map (ppu01230). This branched-chain-amino-acid pathway is **absent in humans**, which is why IPMI is of interest as an antibacterial/antifungal target (Manikandan et al., 2011, P 20938981).

Genomic organization (KEGG genome coordinates). In *P. putida* KT2440 the leucine genes are clustered: - **leuC** = **PP_1985** (large subunit), 2,250,670–2,252,103 - **leuD** = **PP_1986** (this gene), 2,252,100–2,252,744 - **PP_1987**, UbiE/COQ5-family methyltransferase, 2,252,863–2,253,627 - **leuB** = **PP_1988** (3-isopropylmalate dehydrogenase), 2,253,682–2,254,764 - **PP_1984**, LysR-family transcriptional regulator (opposite strand), upstream

Critically, **leuC and leuD overlap by 4 bp** (an ATGA-type overlap), the hallmark of **translational coupling** that ensures the two subunits of the obligate heterodimer are produced in **matched stoichiometry**. The clustering of leuC–leuD with leuB mirrors the *leu* operon organization of enterobacteria and supports co-regulated expression of the pathway.

Regulation (family context). In other bacteria the *leuCD* operon is under end-product (branched-chain amino acid) control — e.g., strong repression by leucine/isoleucine/valine in *Streptomyces coelicolor* (Craster et al., 1999, P 10517590) and dedicated transcriptional regulators in mycobacteria (Rv2989/IclR-like; Angara et al., 2018, P 29523332). The LysR-family regulator adjacent to the *P. putida* cluster (PP_1984) is a plausible local regulator, consistent with this general theme (inference from genomic context; not experimentally proven for *P. putida*).

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7. Evidence That the Function Transfers to the *P. putida* Protein

- **Sequence orthology (bioinformatic).** Global Needleman–Wunsch alignment gives **67.3 % identity (134/199 aligned residues, 93 % coverage) between *P. putida* LeuD (Q88LE7) and experimentally characterized *E. coli* LeuD (P30126)** — far above the ~25–30 % "twilight zone," establishing unambiguous orthology. Alignment to the *M. tuberculosis* crystallographic LeuD fragment gave 43.5 % identity over its length.
- **Structural prediction (AlphaFold).** The AlphaFold DB model AF-Q88LE7-F1 is **highly confident (mean pLDDT 95.9; 93 % of residues >90, 0 % <50)**, describing a single compact swivel domain consistent with IPMI_Swivel (IPR033940). The only region of relatively reduced confidence is an N-terminal loop (~res 30–37, homologous to the *M. tuberculosis* "substrate-discriminating loop"; pLDDT ~84), independently echoing the crystallographic

finding that this specificity loop is the most mobile element of LeuD, while the "substrate-binding loop" region (~res 70–74) is highly ordered (pLDDT ~98).

- **Rule-based annotation.** HAMAP family rule **MF_01031** (curated, expert-derived) assigns the reaction, pathway, subunit composition, and family.
- **Experimental biochemistry** (Salmonella/E. coli): subunit composition, Km, complementation (PMIDs 7026530, 2838459, 374346, 4612005).
- **Structural biology** (M. tuberculosis): fold, substrate loops, two subfamilies (P 20938981).
- **Cofactor biochemistry** (yeast/*B. subtilis*): [4Fe-4S] requirement, cytosolic localization (PMIDs 20097860, 23192348).

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8. Supported and Refuted Hypotheses

Supported: - H1 — *leuD encodes the small subunit of IPMI catalyzing step 2 of leucine biosynthesis.* □ (database + orthology + literature) - H2 — *LeuD is catalytically inert alone and functions only as a LeuC–LeuD heterodimer.* □ (PMIDs 7026530, 2838459) - H3 — *The enzyme is an aconitase-family [4Fe-4S] cytoplasmic hydro-lyase; cluster on LeuC, specificity loops on LeuD.* □ (PMIDs 27929065, 20097860, 23192348, 20938981) - H4 — *In P. putida, leuC/leuD are translationally coupled within a leucine gene cluster.* □ (KEGG coordinates)

Refuted / not supported: - LeuD is **not** an independent catalytic enzyme, **not** the cofactor-bearing subunit, and **not** membrane-associated or secreted. - No evidence for a distinct "same-symbol, different-gene" identity — the annotation is unambiguous.

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9. Limitations and Future Directions

- **No *P. putida*-specific enzymology.** Kinetic parameters (Km, kcat), the exact stereochemistry, and the physiological reversibility have been measured in *Salmonella*/*E. coli*, not in *P. putida* itself; transfer is by strong orthology (67 % identity), which is highly reliable for a conserved primary-metabolic enzyme but remains an inference.

- **No experimental structure of the *P. putida* LeuCD complex.** Structural claims derive from the *M. tuberculosis* LeuD crystal structure and aconitase homology; an AlphaFold model of Q88LE7 could confirm the swivel fold and loop positions.
- **Regulation in *P. putida* is inferred** from genomic context (adjacent LysR regulator; family-wide BCAA end-product control), not directly demonstrated.
- **Secondary EC 4.2.1.35 activity** is a database (KEGG) inference for the orthology group; its physiological relevance in *P. putida* is untested.
- **Essentiality/fitness** under leucine-replete vs. minimal conditions could be confirmed with existing *P. putida* RB-TnSeq datasets.

Key References

- Fultz & Kemper (1981) *J. Bacteriol.* PMID **7026530** — two-subunit composition, Km.
- Stover, Kemper & Marsh (1988) PMID **2838459** — LeuC/LeuD complex; newD.
- Kemper (1974) PMID **4612005** / Fultz, Kwoh & Kemper (1979) PMID **374346** — leuC–leuD complementation, subunit substitution.
- Manikandan et al. (2011) *(structural studies on LeuCD, M. tuberculosis)* PMID **20938981** — crystal structure, substrate loops, subfamilies.
- Watanabe et al. (2016) PMID **27929065** — aconitase superfamily membership.
- Albrecht et al. (2010) PMID **20097860** — [4Fe-4S] activation of IPMI.
- Patil et al. (2012) PMID **23192348** — cytosolic Fe-S IPMI.
- Xu et al. (2004) PMID **15292141** — IPMI substrate flexibility (citraconate route).
- Angara et al. (2018) PMID **29523332**; Craster et al. (1999) PMID **10517590** — leuCD operon regulation (family context).
- Databases: UniProt **Q88LE7** (HAMAP **MF_01031**); KEGG **ppu:PP_1986** / **K01704** / module **M00432**; Rhea **RHEA:32287**.
