Functional Annotation of *ddlB* (D-Alanine—D-Alanine Ligase B, Q88N74) in *Pseudomonas putida* KT2440

Gene / Protein Identity (verified)

Field	Value
Gene symbol	*ddlB*
Ordered locus	PP_1339
UniProt	Q88N74
Protein	D-alanine—D-alanine ligase B (D-Ala-D-Ala ligase B; D-alanylalanine synthetase B)
EC	6.3.2.4
Organism	*Pseudomonas putida* (strain ATCC 47054 / DSM 6125 / KT2440) — PSEPK
Family	D-alanine—D-alanine ligase family (HAMAP-Rule MF_00047)
Domains	ATP-grasp (IPR011761); ATP-grasp subdomain 1 (IPR013815); D-Ala ligase Van conserved site (IPR000291); D-ala_D-ala (IPR005905); Dala_Dala_lig_C (IPR011095)

Identity check: The gene symbol *ddlB*, the EC number 6.3.2.4, the ATP-grasp and D-Ala-D-Ala-ligase domain signatures, and the family assignment are fully mutually consistent. This is unambiguously a member of the D-alanine—D-alanine ligase (Ddl) family. There is no evidence of gene-symbol ambiguity; the only caveat is organism-level: essentially all mechanistic and structural work on this enzyme has been performed on orthologues (*E. coli* DdlB, and Ddl from *M. tuberculosis*, *Y. pestis*, *T. thermophilus*, *T. maritima*, *H. pylori*, enterococci). No study is specific to *P. putida* PP_1339. The function below is therefore assigned to Q88N74 by strong,

unambiguous homology/family inference and validated by biochemistry of well-characterized orthologues, most directly the canonical *E. coli* DdlB, which is the structural reference for the family.

Summary (Answer to the Research Question)

Primary function. *ddlB* encodes **D-alanine—D-alanine ligase B (DdlB, EC 6.3.2.4)**, a cytoplasmic, ATP-dependent ligase of the **ATP-grasp** superfamily. It catalyzes the condensation of **two molecules of D-alanine** into the dipeptide **D-alanyl-D-alanine (D-Ala-D-Ala)**, consuming ATP (2 D-Ala + ATP → D-Ala-D-Ala + ADP + P_i) [PMID 34047462; 35382715]. Its substrate specificity is for D-alanine at both binding subsites; unlike the resistance ligases VanA/VanB, wild-type DdlB strongly disfavours D-lactate/α-hydroxy acids at the second (C-terminal) subsite [PMID 10801495].

Localization. The enzyme functions in the **bacterial cytoplasm**, catalyzing one of the cytosolic steps of peptidoglycan precursor synthesis [PMID 23286234]. Its product, however, is ultimately consumed **extracytoplasmically**: the terminal D-Ala-D-Ala of the completed precursor is the substrate/leaving group for periplasmic penicillin-binding-protein transpeptidases and the direct binding target of glycopeptide antibiotics at the cell surface.

Pathway. DdlB provides the D-Ala-D-Ala dipeptide that the ligase **MurF** adds to UDP-MurNAc-tripeptide, completing **UDP-MurNAc-pentapeptide** (the Park nucleotide) — the cytoplasmic end-product of the Mur pathway and the muropeptide unit later polymerized and cross-linked into the cell-wall sacculus [PMID 34047462; 35382715].

Detailed Findings

1. Reaction catalyzed and substrate specificity

DdlB catalyzes the ATP-dependent ligation of two D-alanine molecules to form D-Ala-D-Ala:

> "D-alanyl-D-alanine ligase (Ddl) is an indispensable adenosine triphosphate-dependent bacterial enzyme … which catalyzes the ligation of two D-alanine molecules into one D-alanyl-D-alanine dipeptide." [PMID 34047462]

The "B" isoform designation reflects the situation in *E. coli* and many other Gram-negatives, where two paralogous ligases, **DdlA** and **DdlB**, both supply D-Ala-D-Ala; either alone is sufficient, and only the **ddlA ddlB double mutant** is a D-Ala-D-Ala **auxotroph**, demonstrating that this dipeptide-forming activity is essential for viability [PMID 15948948]. DdlB was directly identified as "responsible for the condensation of two alanines, forming D-Ala-D-Ala" [PMID 35382715].

The enzyme has **two D-alanine subsites** with distinct affinities. In the *M. tuberculosis* orthologue, K_m,D-Ala1 = 0.075 mM (N-terminal, high-affinity subsite) and K_m,D-Ala2 = 3.6 mM (C-terminal, low-affinity subsite) [PMID 23286234]. The chemistry of subsite 2 is the key determinant of specificity: naturally vancomycin-resistant and Van-type ligases replace an amide-accepting subsite 2 with one that accepts D-lactate (yielding D-Ala-D-Lac); comparison of the D-Ala-D-Lac ligase structure with wild-type **DdlB** revealed "alterations in the size and hydrophobicity of the site for D-lactate binding (subsite 2)" and reduced H-bonding to the second substrate [PMID 10801495]. Wild-type DdlB thus is a true D-Ala:D-Ala (amide-forming) ligase.

2. Structure and catalytic mechanism (ATP-grasp fold)

DdlB is one of the **three founding members of the ATP-grasp superfamily**, alongside biotin carboxylase and glutathione synthetase:

> "The founding members of the family consist of biotin carboxylase, d-ala-d-ala ligase and glutathione synthetase, all of which catalyze the ATP-assisted reaction of a carboxylic acid with a nucleophile via the formation of an acylphosphate intermediate." [PMID 21920581]

Mechanistically, the enzyme follows an **ordered ter-ter** kinetic mechanism: **ATP binds first**, then the two D-Ala substrates bind sequentially [PMID 23286234]. Catalysis proceeds by (i) phosphoryl transfer from ATP to the carboxylate of the first (N-terminal) D-Ala, generating a **D-alanyl-phosphate (acyl-phosphate) intermediate**; (ii) nucleophilic attack by the α-amino group of the second (C-terminal) D-Ala, forming the peptide bond and releasing inorganic

phosphate. General-base chemistry participates in the catalytic step [PMID 23286234]. The intermediate has been captured structurally with **phosphinophosphate transition-state analogs** [PMID 10801495].

The protein is built from **three domains** (N-terminal, central, C-terminal), and catalysis is **conformationally gated**: flexible loops (the "serine loop" recognizing nucleotide phosphates, and the "ω-loop") and rigid-body rotation of the central domain drive an **open → semi-open → closed** transition that sequesters the substrates for chemistry [PMID 26894530; 19770507]. Activity is typically stimulated by monovalent cations (K^+) [PMID 23286234]. Biophysically, recombinant *E. coli* DdlB is a compact, folded enzyme whose stability peaks near its pI (~5.0) [PMID 35382715].

3. Cellular localization and pathway context

DdlB is a **soluble cytoplasmic enzyme** acting in the cytosolic phase of peptidoglycan biosynthesis. Its product feeds directly into the Mur ligase cascade: **MurF** condenses D-Ala-D-Ala onto UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-DAP (UDP-MurNAc-tripeptide) to produce **UDP-MurNAc-pentapeptide** [PMID 34047462]. This nucleotide precursor is then transferred to the lipid carrier (lipid I → lipid II), flipped across the membrane, and polymerized; the **terminal D-Ala-D-Ala** is the acyl-donor recognized by **penicillin-binding-protein transpeptidases** that cross-link glycan strands in the periplasm/cell wall — i.e., the step "required for subsequent extracellular transpeptidase crosslinking of the mature peptidoglycan polymer" [PMID 35382715].

Direct in-cell evidence that DdlB is the committed dipeptide-forming step comes from inhibitor studies: blocking Ddl produces "an increase in D-Ala intracellular pools accompanied by a commensurate decrease in D-Ala-D-Ala" [PMID 30300845].

4. Physiological / pharmacological significance

Because D-Ala-D-Ala is essential and has no human counterpart, Ddl/DdlB is a **validated antibacterial target** [PMID 34047462; 32497961]. The D-alanine analog **D-cycloserine** inhibits Ddl (and alanine racemase), competitively occupying the D-Ala subsites [PMID 23286234; 15948948]. Numerous DdlB-directed inhibitor chemotypes have been developed against the *E. coli* enzyme (diazenedicarboxamides, thiosemicarbazides, hydroxyethylamine phosphonates, flavonoids such as quercetin/apigenin) [PMID 17267218; 30300845; 19196510; 18774266]. The same subsite-2 chemistry that DdlB enforces (amide, not ester) is what glycopeptides (vancomycin) exploit by binding D-Ala-D-Ala directly; Van-type resistance re-routes the

pathway to D-Ala-D-Lac, bypassing this step [PMID 32277698; 10801495]. These points are mechanistically informative for the precise role of PP_1339 in *P. putida*, though *P. putida* itself is not a clinical glycopeptide-resistance model.

Supported vs. Refuted Hypotheses

Hypothesis	Verdict	Basis
Q88N74/PP_1339 is a D-Ala:D-Ala ligase (EC 6.3.2.4) forming D-Ala-D-Ala	**Supported**	Family/domain assignment + orthologue biochemistry [34047462; 35382715]
Reaction proceeds via ATP-grasp, acyl-phosphate intermediate, ordered mechanism	**Supported**	[21920581; 23286234; 19770507]
Enzyme is cytoplasmic; product used downstream (MurF → UDP-MurNAc-pentapeptide → extracellular cross-linking)	**Supported**	[34047462; 35382715; 30300845]
Wild-type DdlB is a D-Ala-D-Lac (Van-type) resistance ligase	**Refuted**	Subsite-2 of DdlB is amide-specific, distinct from VanA/VanB [10801495]

Evidence Quality and Limitations

- **Direct experimental evidence for PP_1339 (Q88N74) itself is lacking**; no *P. putida*-specific enzymology, structure, or knockout was found. The functional assignment rests on (a) unambiguous membership in the D-Ala-D-Ala ligase family via HAMAP MF_00047 and multiple InterPro domain signatures, and (b) deep, consistent biochemical/structural characterization of orthologues — most directly *E. coli* DdlB, the canonical family reference.
- The Ddl family is highly conserved and functionally uniform for the D-Ala:D-Ala reaction; the specificity-determining subsite-2 residues that distinguish D-Ala:D-Ala from D-Ala:D-Lac ligases are well defined, and DdlB-type enzymes are amide-forming. Kinetic parameters (K_m values, K^+ activation) are expected to differ quantitatively in *P. putida* but not qualitatively.

- **Future directions:** direct confirmation for PP_1339 would come from recombinant expression + ADP/phosphate-coupled ligase assays, an AlphaFold/experimental structure superposed on *E. coli* DdlB to verify the two D-Ala subsites and ATP-grasp loops, and complementation of an *E. coli* ddlA ddlB auxotroph.

References (PMIDs)

34047462, 35382715, 21920581, 23286234, 26894530, 19770507, 10801495, 15948948, 30300845, 32497961, 17267218, 19196510, 18774266, 32277698, 17090922.
