

Reconsidering the *in vivo* functions of *Clostridial* Stickland amino acid fermentations

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ARTICLE INFO

Article history:

Received 14 April 2022

Accepted 3 June 2022

Available online 13 June 2022

ABSTRACT

Stickland amino acid fermentations occur primarily among species of *Clostridia*. An ancient form of metabolism, Stickland fermentations use amino acids as electron acceptors in the absence of stronger oxidizing agents and provide metabolic capabilities to support growth when other fermentable substrates, such as carbohydrates, are lacking. The reactions were originally described as paired fermentations of amino acid electron donors, such as the branched-chain amino acids, with recipients that include proline and glycine. We present a redox-focused view of Stickland metabolism following electron flow through metabolically diverse oxidative reactions and the defined-substrate reductase systems, including for proline and glycine, and the role of dual redox pathways for substrates such as leucine and ornithine. Genetic studies and Environment and Gene Regulatory Interaction Network (EGRIN) models for the pathogen *Clostridioides difficile* have improved our understanding of the regulation and metabolic recruitment of these systems, and their functions in modulating inter-species interactions within host-pathogen-commensal systems and uses in industrial and environmental applications.

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1. Introduction

Stickland amino acid fermentations evolved early in the history of life on earth given the abundance of amino acids in planetary ecosystems [1]. In 1934, Stickland identified paired oxidative and reductive fermentation of amino acids in *Clostridium sporogenes* that supported anaerobic growth in the absence of glucose [2]. Oxidative Stickland reactions remove electrons from donor amino acids (Fig. 1A) [3]. Donors such as alanine and the branched chain amino acids leucine, isoleucine and valine start with two more hydrogens than their cognate keto acids [4], while donors such as threonine or methionine have equivalent oxidation states with their keto acids [3]. The Stickland reductive reactions transfer electrons to an acceptor amino acid, commonly proline [5], glycine [6,7], and also to leucine [8]. In aggregate, the Stickland systems provide redox reactions that can be recruited to support changing needs in cellular metabolism.

Stickland metabolism is a hallmark of the Cluster XI *Clostridia*, which includes *Clostridioides difficile* and *Paraclostridium bifermentans* that carry the proline, glycine, and reductive leucine systems (Fig. 2) [5,8,9]. Other clusters including many, but not all, species of Cluster I (*C. sporogenes*, *C. botulinum*), Cluster II (*H. histolyticum*), and Cluster XIVa (*C. scindens*) carry one or more reductive systems (Fig. 2). Outside of the *Clostridia*, these systems are uncommon but occur in other branches of phylum Firmicutes and some Archaea [10–13].

Multiple genes comprise each Stickland reductase, enzymes that have defined substrate specificities. The glycine reductase genes *grdABC* encode a selenoenzyme and thioredoxins *trxA* and *trxB* (Figs. 1B and 2B) [7] that metabolize glycine to acetate and ammonia [14]. Some species carry multiple *grdB* or *grdX* homologs to ferment chemically related substrates such as sarcosine and betaine (Fig. 2B) [6,15]. The *had* genes encode enzymes that reduce leucine to isocaproate, with electron handling via *eftA1* and *eftB1* (Figs. 1C and 2C) [8]. Homologous systems in other *Clostridia* reduce alanine, glutamate, caffeate, or phenylalanine to their derivative carboxylates [16,17]. The proline reductase's *prdABC* genes encode a selenoenzyme that metabolizes proline to 5-aminovalerate and

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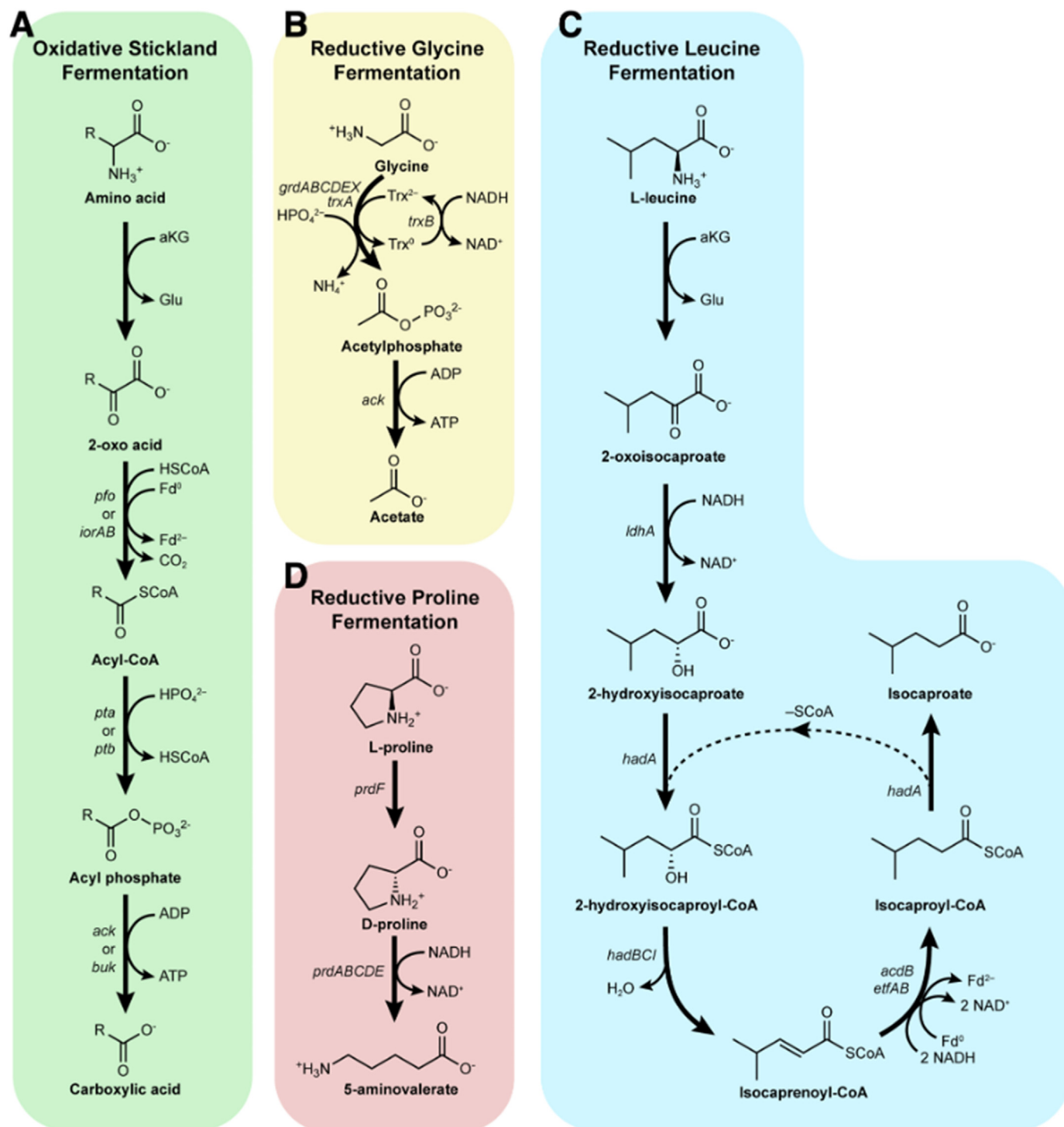


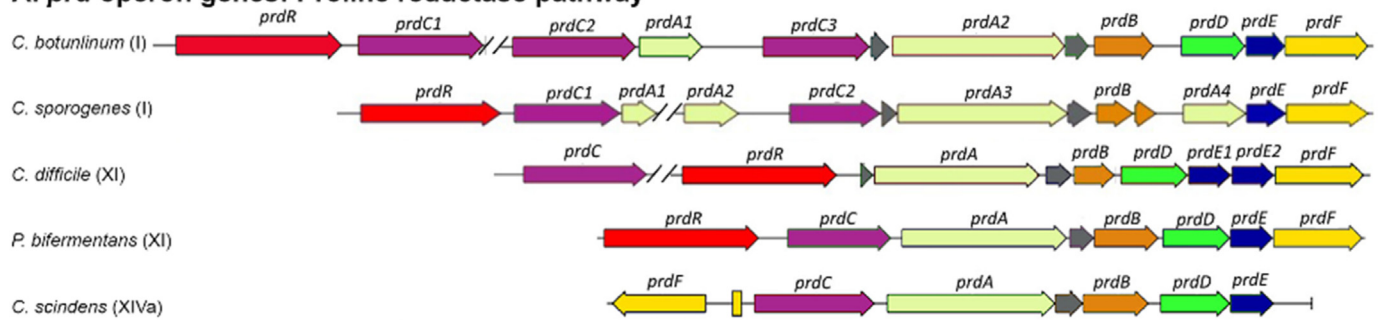
Fig. 1. Clostridial Stickland fermentation pathways. **Legend:** (A) Oxidative Stickland fermentations generate one ATP and yield two low-potential electrons to ferredoxin (Fd) or flavodoxin per amino acid; R = amino acid side group. (B) Glycine reductase oxidizes one equivalent of thioredoxin (Trx) to yield ammonium, acetate, and ATP. (C) Reductive leucine metabolism in *Clostridioides difficile* oxidizes three equivalents of NADH and reduces one equivalent of ferredoxin, yielding isocaproate. Equivalent pathways in other *Clostridia* reduce alanine to propionate (*C. propionicum*), glutamate to butyrate (*C. symbiosium*), or phenylalanine to phenylpropionate (*C. sporogenes*) [65]. (D) Proline reductase oxidizes one equivalent of NADH to NAD⁺, yielding 5-aminovalerate.

couples electron transfer with the bacterial Rnf system (Figs. 1D and 2A) [5]. Originally named for its role in *Rhodobacter* nitrogen fixation, this system operates broadly among bacteria to generate ion gradients and regenerate electron carriers [4,18].

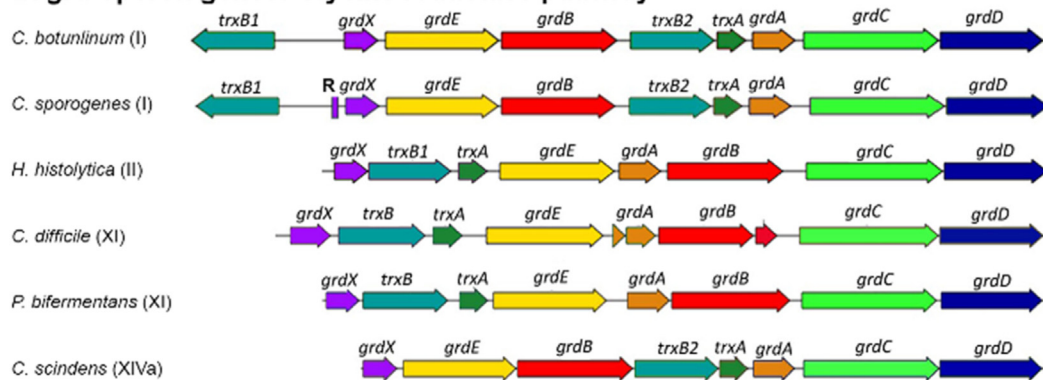
In contrast to the substrate specificity of the reductive pathways,

the known oxidative Stickland pathways have greater promiscuity. Oxidative metabolism of alanine produces acetate via alanine's deamination to pyruvate and oxidation via pyruvate:ferredoxin oxidoreductase (*pfo*) [19], a pathway that other pyruvate-generating amino acids, such as cysteine and serine, may follow.

A. *prd* operon genes: Proline reductase pathway



B. *grd* operon genes: Glycine reductase pathway



C. *had* operon genes: Reductive leucine pathway

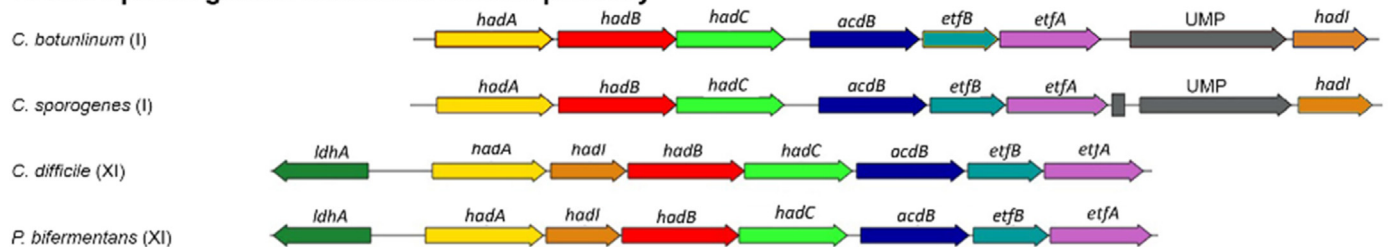


Fig. 2. Genetic organization of the Stickland pathways among *Clostridia* clusters. **Legend:** Stickland reductase loci across *Clostridia* identified using BLAST [13,66] to the *C. difficile* and *P. bifermentans* *prd*, *grd*, and *had* operon genes. Cluster 1: *C. botulinum* ATCC3502 and *C. sporogenes* ATCC3584; Cluster II: *Hathawayia histolytica* NCTC503 (formerly *Clostridium histolyticum*); Cluster XI: *C. difficile* ATCC43255 and *P. bifermentans* DSM638; Cluster XIVa: *Clostridium scindens* ATCC35704. A: *prd* proline-reductase genes: *prdR*: transcriptional activator; *prdF*: proline racemase; *prdA* *prdB* and *prdB*: selenoenzyme subunits; *prdB*: di-thiol stabilizing protein; *prdB*: putative stabilizing protein; *prdB*: putative stabilizing protein; *prdB*: putative stabilizing protein; *prdB*: putative stabilizing protein. *H. histolytica* lacks a homologous locus. Putative ORFs with unknown functions shown in gray. B: Glycine reductase (*grd*) genes. The *grdAB*: selenoenzyme subunits that complex with *grdE* and the *grdC* and *grdD* and may include *grdX*; *trxB*: thioredoxin; *trxB* genes: thioredoxin reductases; The R for *C. sporogenes* indicates a putative glycine-responsive riboswitch. C: Reductive leucine pathway: *ldhA*: 2-hydroxyisocaproate dehydrogenase; *hadA*: 2-hydroxyisocaproate CoA transferase; *hadI*: dehydratase activator; *hadB* and *hadC*: (R)-2-hydroxyisocaproyl-CoA dehydratase; *acdB*: acyl-CoA dehydrogenase; *etfA* and *etfB*: electron transfer flavoproteins. *C. scindens* and *H. histolytica* lack a homologous locus.

Indolepyruvate:ferredoxin oxidoreductase (*iorAB*) metabolizes tryptophan and potentially other aromatic amino acids [20]. The genes and enzyme systems supporting oxidative metabolism of other amino acids remain ill-defined in many species [9,21,22] but exist per known oxidative products such as isovalerate from leucine and isobutyrate from valine [23,24].

Each Stickland system variably conserves cellular carbon and nitrogen. 5-aminovalerate production may excrete 100% of proline's carbon and nitrogen. In contrast, ammonia and acetate from glycine metabolism may be excreted or shunted into pathways producing ethanol, or pyruvate via fixation of CO₂ with acetate by pyruvate:ferredoxin oxidoreductase [25]. Oxidative leucine metabolism deaminates and decarboxylates the leucine, releasing isovalerate, while reductive leucine metabolism can excrete 100% of the carbon backbone as isocaproate [8,23]. While a seeming loss of

cellular carbon, these metabolites have potential extracellular benefit to Stickland fermenters, particularly within dense ecosystems where they may cross-feed other species that provide supporting nutrients [23].

2. Energetic contributions of Stickland metabolism

Stickland-fermented amino acids are relatively weak electron acceptors with reduction potentials between −190 mV (mV) and −10 mV, as compared to an endpoint of +810 mV for electron flow to molecular oxygen [26,27]. Nevertheless, they serve as an important electron sink under anoxic conditions and in the absence of stronger oxidizing agents [8,16,28,29]. In supplementing substrate-level phosphorylation on the oxidative branch (Fig. 3A), the Stickland reductases optimize further energy capture from

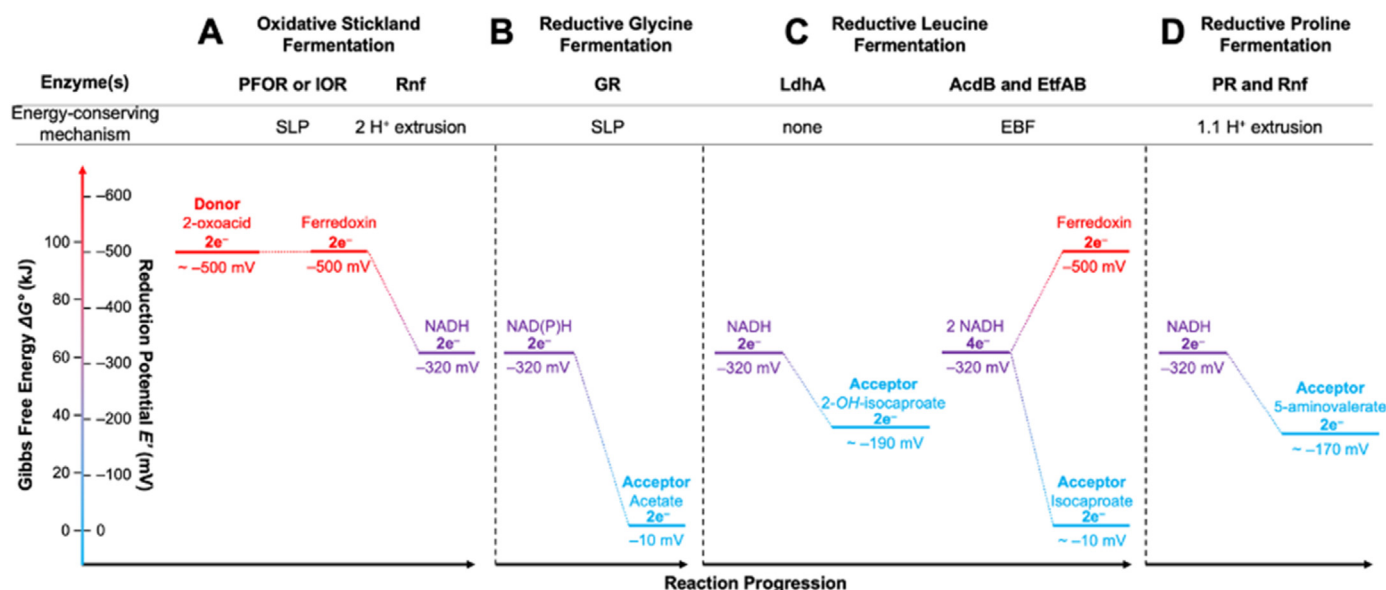


Fig. 3. Reduction potential of electrons in *C. difficile*'s Stickland systems. **Legend:** Redox potentials of species pairs from Buckel et al. [67] and Thauer et al. [68]. (A) The prototypical oxidative half-reaction of Stickland metabolism transfers two low-potential electrons from a 2-oxoacid donor to ferredoxin by enzymes such as pyruvate:ferredoxin oxidoreductase (PFOR) or indolepyruvate:ferredoxin oxidoreductase (IOR), with substrate-level phosphorylation (SLP) by coupled phosphotransacylase and acyl kinase reactions. The electrons held by ferredoxin may be transferred to NADH by the Rnf complex, with concomitant extrusion of one proton per electron transfer. (B) The glycine reductase (GR) system transfers two electrons from NADH or NADPH to glycine, forming acetylphosphate, with substrate-level phosphorylation by acetate kinase. (C) In the reductive leucine pathway (R)-2-hydroxyisocaproate dehydrogenase (LdhA) first reduces 2-oxoisocaproate to (R)-2-hydroxyisocaproate (2-OH-isocaproate), with NADH as the electron donor and no known energy salvage mechanism. Then, an acyl-CoA dehydrogenase (AcdB) reduces isocaprenyl-CoA to isocaproyl-CoA using two electrons from NADH while an electron transfer system (EtfAB) promotes two electrons from NADH to ferredoxin in an electron-bifurcating mechanism (EBF). (D) Proline reductase (PR) reduces D-proline to 5-aminovaleate. Rnf coupling extrudes approximately 1.1 protons per molecule of proline. The standard Gibbs free energy of formation ΔG° for the proline reductase reaction was calculated using Hess's Law and the estimated standard ΔG of formation for the individual species from MetaCyc [69], then converted to standard redox potential using the equation $E^\circ = \Delta G^\circ / (-nF)$, where $n = 2$ electrons transferred and F is Faraday's constant. The standard redox potential of the proline and 5-aminovaleate pair was calculated by subtracting the redox potential contribution of NADH/NAD⁺, 320 mV, from the reaction standard redox potential.

electrons via mechanisms including substrate-level phosphorylation, as with glycine reductase (Fig. 3B), electron bifurcation reactions in the reductive leucine pathway (Fig. 3C), or Rnf complex coupling with proline reductase (Fig. 3D). Genetic and biochemical studies have shown other direct interactions among electron bifurcating reactions and the Rnf system [30], creating a potential “anaerobic reductosome” to coordinate energy-generation from pathways such as butyrate production from glycolytic, ethanolamine, or Wood-Ljungdahl acetogenic reactions [4,31].

Thus, a more nuanced view of Stickland metabolism considers recruitment of the oxidative and reductive half reactions relative to energetic needs, substrate and electron carrier availability, and local redox potential. Reductive metabolism of abundant amino acids can support high-flux energy-generating pathways, such as oxidative glycolytic reactions, by regenerating electron carriers with additional energy harvest (Fig. 3). Conversely, oxidative metabolism of amino acids for energy can be balanced with other forms of reductive metabolism such as mixed acid fermentations converting pyruvate to lactate or to ethanol. Nisman et al. alluded to this modularization in 1948, noting coupling of glycine's reduction to acetate with oxidative reactions of non-amino acid substrates including glucose, pyruvate, or acetaldehyde [32,33].

3. Genomic systems supporting Stickland metabolism

Stickland fermenters harbor additional machinery to transform exogenous amines into fermentable substrates. Nearly all Stickland fermenters are proteolytic, identified microbiologically by rapid degradation of proteins such as gelatin or proteolytic activity in *Clostridial* meat-granule media [34], and demonstrated genomically by multiple excreted proteases and peptide and amino acid

transport systems [23], including the *brnQ*-family transporters for branched chain amino acids [35] and *Clostridial* *app* and *opp* oligopeptide transport systems [23,36]. Gut-colonizing species harboring a proline reductase also commonly co-express a 4-hydroxyproline dehydratase (*pflD*) and pyrroline-5-carboxylate reductase (*proC*) to convert 4-hydroxyproline, a breakdown product of animal-origin collagen, to proline [37,38], illustrating how host and dietary factors feed Stickland fermenters [23]. Some species also metabolize ornithine to proline and alanine, providing a robust redox pair for Stickland metabolism that has been associated with rapid biomass expansion [23,39]. Purinolytic species of *Clostridia* metabolize xanthine to formiminoglycine and subsequently to glycine to support reductive glycine metabolism [40], again leveraging substrates that may be abundant in dense ecosystems such as the gut, midden heaps, or anaerobic marine and riparian sediments. Some species further process Stickland metabolites for energetic and non-energetic purposes, such as aldehyde/alcohol and lactate dehydrogenases that reduce Stickland metabolites to regenerate NAD⁺ [31], and *C. difficile*'s HpdBCA decarboxylase (*hpdBCA*) that converts the Stickland tyrosine metabolite 4-hydroxyphenylacetate to *p*-cresol, a metabolite shown to have anti-microbial activities against some Gram negative species and potential influence on a host's dopaminergic pathways, to the level of altering phenotypic behaviors [29,41–46].

4. Regulatory control of the Stickland reductase systems

In the presence of proline, *C. difficile*'s PrdR proline reductase regulator induces *prd* gene expression and inhibits glycine reductase expression (Fig. 2) [47,48]. Indirect effects have also been demonstrated by Rex, the sensor of NAD⁺/NADH balance and

cellular redox state [47]. However, little is known about the transcriptional regulation of reductive leucine metabolism, or how *C. difficile* and other *Clostridia* coordinate transcriptional regulation among their Stickland reductase systems relative to changing conditions.

In vitro and *in vivo* analyses of *C. difficile* have supported new Environment and Gene Regulatory Interaction Networks (EGRIN) to define co-regulatory gene modules and transcription factors controlling the pathogen's conditional behaviors, including those seen *in vivo* with protective versus disease-promoting commensals [49]. Model predictions inferred the partitioning and differential expression of the Stickland reductase genes, particularly separation of proline reductase genes from the glycine and reductive leucine pathways (Fig. 4A; <http://networks.systemsbiology.net/cdiff-portal/>). Members of the *grd* and *had* reductase systems co-occur in EGRIN modules, #10, 129, 147 and 159, indicating capacity for

co-expression of the reductase systems, and with genes involved in fatty acid metabolism, electron transport, and the Wood-Ljungdahl pathway (Fig. 4A). EGRIN further inferred coordinate regulation at the level of three transcriptional regulators, PrdR, CD630_16930, and the sigma factor SigL (Fig. 4A–B) with predicted inputs from 17 additional transcription factors, including CcpA, FapR and SigD (Fig. 4C). EGRIN's predictions correlate with known effects of the PrdR and CcpA regulators on the proline and glycine reductase systems [47,50] and SigL-mediated expression of the reductive leucine and other amino acid degradation pathways [51,52]. Model predictions further inferred regulation by CD630_16930, an ArsR-family transcription factor [53] located in a genetic locus adjacent to a thioredoxin gene system. Given the existence of these regulator homologs in other Stickland-fermenting *Clostridia*, these loci provide starting points to explore regulation of the reductases in other species of clinical, industrial, and environmental importance.

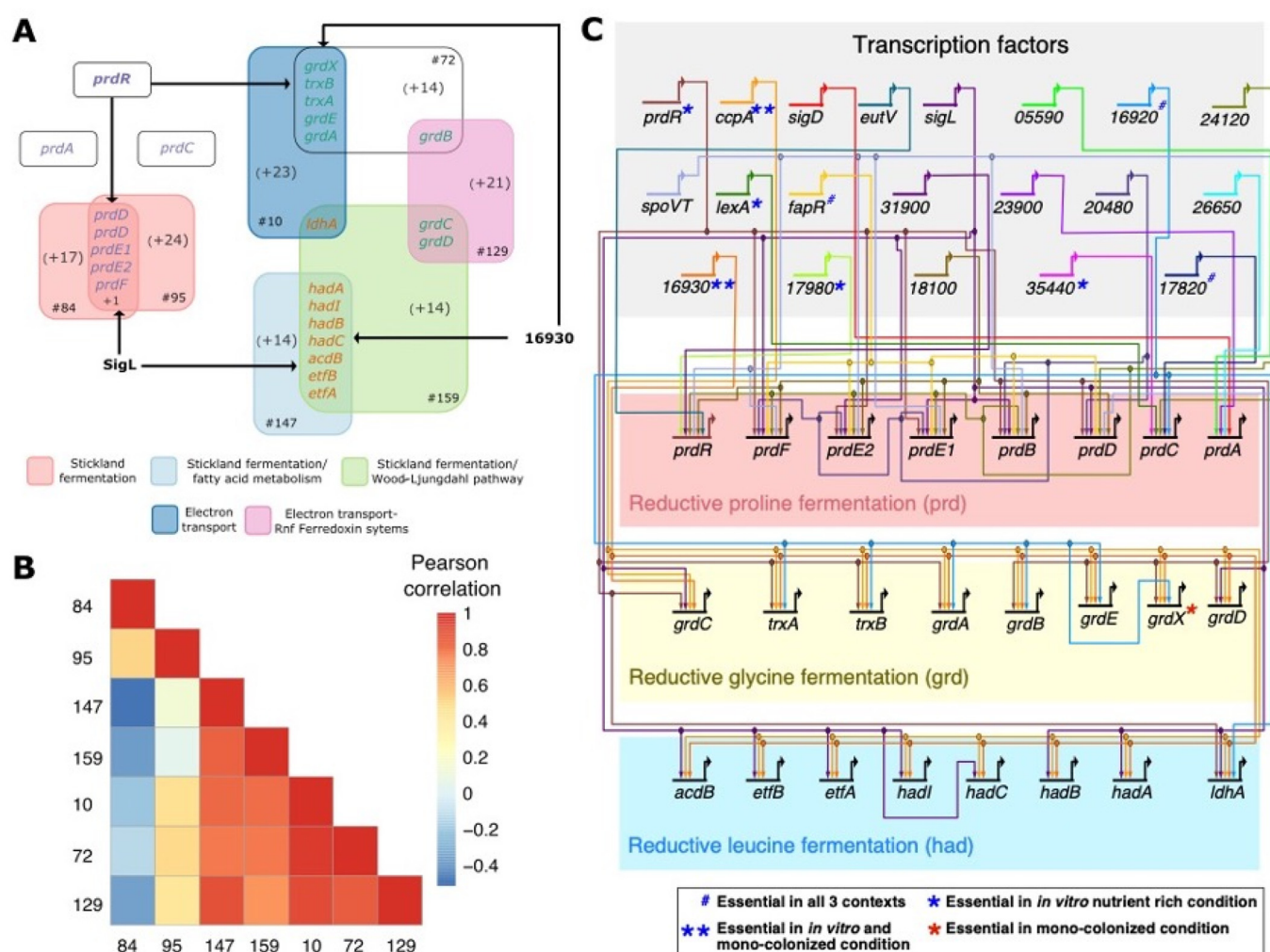


Fig. 4. EGRIN module and co-regulatory predictions for *C. difficile*'s Stickland reductase systems. Legend: EGRIN modules containing the *prd*, *grd* and *had* genes were defined in Arrieta-Ortiz et al. [49] and are available through the *C. difficile* Portal at <http://networks.systemsbiology.net/cdiff-portal/>. (A) Conditional partitioning of Stickland reductase systems across EGRIN modules of co-regulated genes. Only Stickland reductase system genes are shown. Module ID number (starting with the '#' symbol) is indicated for each EGRIN module that has more than one gene of interest. The number of additional non-reductase genes in each module is indicated with the '+' notation, such as (+24) for module #95. Gene modules are color-coded according to their functional enrichment. The three regulators with the highest number of target EGRIN modules among the displayed modules were included: PrdR, SigL and CD630_16930. (B) Pearson correlations calculated across the *C. difficile* transcriptional compendium [49] among the EGRIN modules in panel (A) showing positive correlations among *grd*- and *had*-associated modules, but weak to negative associations with the *prd*-associated modules #84 and 95. (C) Biotapestry [70] visualization of the inferred transcriptional network for the Stickland reductase systems. Regulators without common gene names are shown using the CD630 nomenclature ("CD630_#####").

5. Contributions of Stickland metabolism on inter-species interactions and in industrial applications

The Stickland pathways support high flux redox metabolism for commensal and pathogenic *Clostridia*, particularly during early colonization of anaerobic host ecosystems [23,54]. The amino acids fermented in Stickland reductive pathways are notably abundant in host-origin mucins [55], providing a source of reductive substrates along the cephalocaudal axis of the gut. Differential recruitment of the Stickland reductive systems in *Clostridioides difficile* and in the protective Stickland-fermenting commensal, *Paraclostridium bifermentans* modulate critical events early in the pathogen's colonization to impact host outcomes from infection [23,49]. Host protection against *C. difficile* infection has also been reported with the Stickland-fermenting Cluster XI species *Clostridium hiranonis*, and Cluster XIVa species *Clostridium scindens* [56,57]. Preventive and therapeutic considerations in *C. difficile* disease may thus consider the effects of disease-triggering antibiotics that indiscriminately target commensal Stickland fermenters, and mechanisms to support their recovery, including via bacteriotherapeutic re-introduction, modulation of dietary or host-origin fermentable substrates, or to support other commensals to enhance their recovery within disrupted intestinal ecosystems [23,56]. Targeting of the Stickland pathways may also be considered for other *Clostridial* pathogens including *C. botulinum* and for associated applications in food safety.

The Stickland metabolites from oxidative aromatic amino acids have known bioactive properties in complex host-microbiota ecosystems, including inhibition of other microbial species [58], and neuroendocrine activities on host tissues [23,44–46]. In mouse monocolonization studies with *Paraclostridium bifermentans*, for instance, colonization increases gut concentrations of tryptamine and tyramine [23], degradation products of tryptophan and tyrosine, respectively, which have known neuroendocrine effects on gut functioning, heart rate, and in the central nervous system [59,60].

Stickland fermenters support metabolic processes in other settings, particularly in anoxic sediments and in methane and biofuel production [61,62]. Stickland fermenters have capacity to produce substrates of industrial use, such as 5-aminovaleate for polyamide synthesis [63], and from broader input feedstocks than aerobic strains may use. Many products of oxidative aromatic amino acid fermentations produce substrates such as phenylacetate and toluene that have broader chemical and industrial uses [64]. Thus, defining the metabolic inputs and gene regulatory networks to modulate induction of these systems has broad applicability in how we interact with commensal and pathogenic Stickland species, and their constructive use in diverse applications.

Funding

This work was supported by R01AI153605 and P30DK034854 from the National Institutes of Health to LB, R01AI128215, R01AI141953, and U19AI135976 from NIH and IIBR-2042948 from the National Science Foundation to NSB.

Declaration of competing interest

LB is an inventor on patents for *C. difficile* microbiota therapeutics, and is the founder, SAB chair and a stock holder in ParatoBio Inc. Remaining authors declare no competing interests.

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