Extracellular electron transfer powers flavinylated extracellular reductases in Gram-positive bacteria

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Mineral-respiring bacteria use a process called extracellular electron transfer to route their respiratory electron transport chain to insoluble electron acceptors on the exterior of the cell. We recently characterized a flavin-based extracellular electron transfer system that is present in the foodborne pathogen *Listeria monocytogenes*, as well as many other Gram-positive bacteria, and which highlights a more generalized role for extracellular electron transfer in microbial metabolism. Here we identify a family of putative extracellular reductases that possess a conserved posttranslational flavinylation modification. Phylogenetic analyses suggest that divergent flavinylated extracellular reductase subfamilies possess distinct and often unidentified substrate specificities. We show that flavinylation of a member of the fumarate reductase subfamily allows this enzyme to receive electrons from the extracellular electron transfer system and support *L. monocytogenes* growth. We demonstrate that this represents a generalizable mechanism by finding that a *L. monocytogenes* strain engineered to express a flavinylated extracellular urocanate reductase uses urocanate by a related mechanism and to a similar effect. These studies thus identify an enzyme family that exploits a modular flavin-based electron transfer strategy to reduce distinct extracellular substrates and support a multifunctional view of the role of extracellular electron transfer activities in microbial physiology.

bacterial pathogenesis | cellular respiration | electromicrobiology | exoelectrogen | fumarate/urocanate

Energy metabolism generates the ATP that fuels most cellular processes and thus represents one of the most important activities in biology. Compared to their multicellular counterparts, microbes adopt considerably more variable strategies of energy metabolism. This diversity is largely due to the fact that metabolic strategies have been evolutionarily optimized to exploit a particular ecological niche. This means that microbial mechanisms of energy metabolism fundamentally reflect important physicochemical features of the environments in which they are utilized. Addressing the basis of metabolism is thus important for understanding basic microbial growth mechanisms and the nature of complex, often polymicrobial, ecosystems.

Heterotrophic microbes employ both fermentative and respiratory mechanisms of energy metabolism. These processes fundamentally differ in electron acceptor usage and/or ATP synthesis mechanisms. In cellular respiration, electrons from a donor are transferred to a terminal acceptor through a series of intermediates within the cell membrane. The resulting electron transport chain maintains redox homeostasis by regenerating nicotinamide adenine dinucleotide (NAD+) and can generate a proton gradient that powers oxidative phosphorylation.

In contrast to metazoans, which typically use molecular oxygen as a respiratory electron acceptor, microbes residing in anoxic environments exploit a variety of energetically favorable compounds. Some bacteria route their electron transport chain to the exterior of the cell in a process called extracellular electron transfer (1). This allows for the respiration of compounds that are inaccessible in the cell membrane, such as insoluble mineral oxides (2, 3). While extracellular electron transfer has primarily been studied in the context of specialized mineral-respiring bacteria, we recently identified an 8-gene locus that confers the foodborne pathogen *Listeria monocytogenes* with extracellular electron transfer activity (4). Our findings are supported by complementary studies by others and suggest that the products of these genes achieve extracellular electron transfer through a flavin-based transfer mechanism that is conserved in many other Gram-positive bacteria (4–8). This system is characterized by a lipoprotein, FmnB, which is an ApbE-like extracellular flavin transferase that posttranslationally flavinylates (i.e., covalently attaches a flavin mononucleotide group) the lipoprotein PplA (4). Extracellular electron transfer seems to result from electron transfer by an NADH dehydrogenase, Ndh2, to a quinone in the membrane, from the quinone to surface-associated flavinylated PplA, and finally to the terminal electron acceptor (which can be free flavins, ferric iron, and, potentially, other redox-active small molecules) (4).

Here we identify branches to the extracellular electron transport chain that direct electrons to a family of reductases. These reductases promote growth on distinct small molecule electron acceptors, including fumarate and urocanate. Mechanistic insights reveal an unusual extracellular site of reduction and conserved flavinylation motif that seems to provide a modular mechanism for segregating electron flux to distinct electron

Significance

Strategies of energy metabolism are variable across the microbial world and fundamentally reflect distinct ecological dynamics. The complex electron transfer mechanisms that support many such processes take place in the cell membrane but can be wired to the exterior of the cell in some circumstances. The results presented here reveal a family of extracellular reductases that possess a post-translationally flavinylated sequence motif. This motif provides a conserved framework that mediates electron transfer from the membrane to different extracellular small molecules. These findings advance our understanding of microbial energy metabolism and identify a generalized and modular electron transfer strategy that could be exploited for applications in metabolic engineering.

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acceptors. This system is conserved in numerous Gram-positive bacteria, including many that have been considered to chiefly adopt fermentative growth strategies. These findings thus provide new insight into the basis of extracellular electron transfer activities and the nature of the ecological niche occupied by many Gram-positive bacteria.

Results

Identification of a Class of Extracellular Fumarate Reductases. We previously found that *L. monocytogenes* will grow on sugar alcohol substrates if an electron acceptor (oxygen or ferric iron) is present (4). To identify novel *L. monocytogenes* electron acceptor usage capabilities, we screened a number of common microbial electron acceptors for their ability to stimulate anaerobic growth on the sugar alcohol xylitol. Of those tested, only the inclusion of fumarate was comparable to ferric iron in promoting *L. monocytogenes* growth (Fig. 1*A*). Consistent with fumarate acting as an electron acceptor under these conditions, we observed that provision of fumarate led to an accumulation of the reduced product succinate in the growth medium (Fig. 1 *B* and *C*).

In *L. monocytogenes*, the gene *lmo0355* (henceforth referred to as *frdA*) encodes a predicted lipoprotein that was previously proposed to possess fumarate reductase activity (9, 10). Consistent with FrdA representing the sole fumarate reductase in *L. monocytogenes*, we found that a Δ*frdA* strain failed to produce succinate and did not grow on xylitol/fumarate medium (Fig. 1 *A* and *C*).

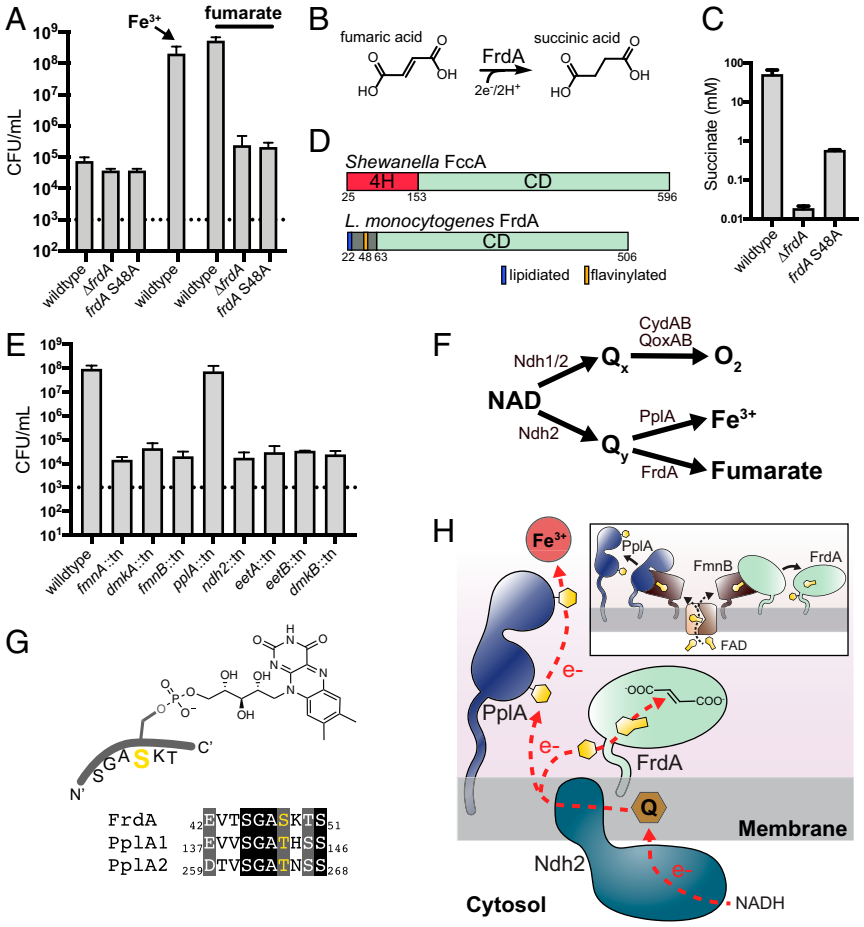

Fig. 1. The *L. monocytogenes* FrdA is a flavinylated extracellular fumarate reductase. (*A*) The effect of electron acceptors on *L. monocytogenes* growth with xylitol as the electron donor. The CFU/mL after a 48-h incubation period are shown. The inoculum CFU are represented with a dashed line. Results ($n = 3$) are expressed as means and SDs. (*B*) Reaction catalyzed by fumarate reductase. (*C*) Succinate concentration in the supernatant of *L. monocytogenes* strains cultivated in medium supplemented with fumarate. Results ($n = 3$) are expressed as means and SDs. (*D*) Domain layout of signal peptide-processed fumarate reductases from *L. monocytogenes* and *Shewanella* sp. (as inferred from the crystal structure, PDB 1D4D). Catalytic domains (CD), which share 45% identity and 64% similarity between the two enzymes, are colored green. Distinct N-terminal domains are colored gray and red, with the 4H indicating the 4 hemes bound by the *Shewanella* enzyme. (*E*) Growth of previously identified extracellular electron transfer mutants (4) on xylitol/fumarate medium. Results ($n = 3$) are expressed as means and SDs. (*F*) Putative electron transfer pathways in *L. monocytogenes*. The Q_x and Q_y refer to unidentified quinones used for aerobic and anaerobic respiratory capabilities. Distinct lipoproteins appear to be responsible for directing electron flux toward extracellular electron transfer and fumarate reductase. (*G*) The FrdA flavinylation site. Flavinylated FrdA peptide is schematically depicted in the *Top* half of the panel. The side chain of the modified serine and the anticipated posttranslational modification are shown. In the *Bottom* part of the panel, the amino acid sequence of putative flavinylation sites in *L. monocytogenes* FrdA and PplA proteins are aligned. Posttranslationally modified residues are colored yellow. Black highlights conserved and gray similar residues within the three sites. (*H*) Model of branched extracellular electron transfer to FrdA and PplA. The *Inset* shows the proposed mechanism of flavinylation. A transporter secretes flavin adenine dinucleotide that FmnB uses as a substrate to modify lipoproteins PplA and FrdA. The extended yellow hexagon represents flavin adenine dinucleotide and the simple yellow hexagon represents the flavinylated posttranslational modification. In the main panel, dashed red arrows track the proposed course of electron travel. Ndh2 transfers electrons from NADH to a quinone (Q) derivative. Electrons are then transferred to flavinylation sites on PplA or FrdA and finally to respective terminal electron acceptors. Electron transfer to fumarate is likely facilitated by a flavin adenine dinucleotide cofactor noncovalently bound to FrdA.

Since we and others previously confirmed that FrdA is associated with the surface of the cell (4, 11, 12), we conclude that this enzyme is the founding member of a family of extracellular fumarate reductases.

Extracellular Fumarate Reductase Interfaces with the Extracellular Electron Transfer Apparatus. While characterized fumarate reductases possess similar catalytic domains that bind a flavin adenine dinucleotide cofactor (covalently or noncovalently) (13–17), these enzymes are distinguished by distinct cellular localizations and electron transfer mechanisms. Soluble cytoplasmic fumarate reductases in *Saccharomyces cerevisiae* and *Klebsiella pneumoniae* use NADH and an unidentified electron donor, respectively (18–21). Fumarate reductases in *E. coli* and *Wolinella succinogenes* engage the cytoplasmic side of a multiprotein inner membrane complex and receive quinol electrons via a series of iron-sulfur clusters (22–25). By contrast, *Shewanella* species have a soluble periplasmic enzyme called FccA, which has an N-terminal domain that receives electrons from a quinol oxidase and then transfers these to the C-terminal fumarate reductase catalytic domain through a series of bound heme molecules (26–29) (Fig. 1*D*).

The extracellular localization of *L. monocytogenes* FrdA means that this enzyme likely receives electrons by a unique mechanism. The observation that multiple Firmicutes genomes include a gene homologous to *frdA* on a genetic locus that also contains core extracellular electron transfer genes (*SI Appendix*, Fig. S1) suggested a functional connection to extracellular electron transfer. To determine whether the core extracellular electron transfer apparatus was responsible for electron transfer to the *L. monocytogenes* FrdA, we tested mutants in all 8 genes on the locus associated with extracellular electron transfer for growth on xylitol/fumarate medium. With the notable exception of *pplA*, the other seven mutants failed to grow on fumarate (Fig. 1*E*). Since PplA is a surface-associated flavoprotein that is likely responsible for terminal electron transfer to extracellular acceptors (4), these results are consistent with the initial electron transfer steps being shared between fumarate and ferric iron reduction, with the final transfer to fumarate bypassing PplA in favor of FrdA (Fig. 1*F*).

A Conserved Flavinylation Site Is Essential for Extracellular Fumarate Reduction. While growth assays established that the components of the extracellular electron transfer apparatus route electrons to FrdA, the divergent phenotypes for *fmnB*::tn and *pplA*::tn mutants (Fig. 1*E*) stimulated additional questions about the mechanism of electron transfer. We previously found that FmnB is an ApbE-like extracellular flavin mononucleotide (FMN) transferase that posttranslationally flavinylates PplA (4). Because the divergent fumarate growth phenotypes for *fmnB*::tn and *pplA*::tn mutants meant that the *fmnB*::tn growth phenotype could not be explained by a loss of PplA function, we reasoned that FmnB likely acted on a second protein substrate essential for fumarate reduction.

As FrdA seemed to present the most logical target for posttranslational flavinylation, we assayed flavin transferase activity using recombinantly expressed proteins. Consistent with the hypothesis, intact protein mass spectrometry measurements confirmed that incubation with FmnB resulted in the covalent attachment of a single FMN group to FrdA (*SI Appendix*, Fig. S2*A*). Subsequent LC-MS/MS analysis of proteolytically digested FrdA identified Ser48 as the sole site of flavinylation (Fig. 1*G* and *SI Appendix*, Fig. S2*B*).

Several observations are consistent with FrdA Ser48 representing a physiologically relevant flavinylation site. First, the amino acid sequence surrounding Ser48 is strikingly similar to established motifs targeted by FmnB and related ApbE-like enzymes (Fig. 1*G*) (30–32). Second, in analogy to the *Shewanella* fumarate reductase FccA, which uses an N-terminal domain with redox-active hemes to transfer electrons to the enzyme active site (26–29), Ser48 is situated on the N-terminal region between the lipidation site and the fumarate reductase domain (Fig. 1*D*). Finally, sequence alignments reveal that Ser48 is conserved in putative fumarate reductase lipoproteins from a number of Gram-positive bacteria that possess extracellular electron transfer genes and thus might be expected to reduce fumarate via a conserved mechanism (*SI Appendix*, Fig. S3).

To address the functional significance of Ser48 flavinylation, we generated an *L. monocytogenes* strain with a FrdA Ser48 to alanine (S48A) point mutation. Consistent with Ser48 representing a functionally important residue, this mutation substantially decreased succinate production and prevented growth on xylitol/fumarate medium (Fig. 1 *A* and *C*). We thus conclude that flavinylation of Ser48 is essential for fumarate reductase activity and propose that this modification mediates electron transfer from components in the membrane to the noncovalently bound flavin adenine dinucleotide cofactor at the active site (Fig. 1*H*).

Extracellular Electron Transfer Genes Exhibit a Fumarate Reductase-Independent Role in Intestinal Colonization. *L. monocytogenes* cause foodborne disease in humans and the ability to compete with gut microbiota to efficiently colonize the intestinal mucosa is an important virulence phenotype. We previously found that a *L. monocytogenes ndh2*::tn mutant exhibited a defect in colonization of the murine gut, as measured by colony-forming units shed in feces (4). Since the studies described in the preceding sections revealed that, in addition to its role in extracellular electron transfer, Ndh2 was also required for fumarate reduction, we sought to clarify the biologically relevant electron acceptor used in the context of the gastrointestinal tract.

To investigate the role of fumarate reduction in the ability of *L. monocytogenes* to survive in the gastrointestinal tract, we chose to use a mouse strain (BALB/cByJ) that is more susceptible to *L. monocytogenes*. This allowed us to directly measure the amount of *L. monocytogenes* in the gut without the need for antibiotic pretreatment, which can alter the gut microbiota by depleting butyrate-producing *Clostridia* and increasing oxygenation at the intestinal epithelium (33).

Competition studies confirmed that mutants with impairments in posttranslational flavinylation (*fmnB*::tn) or the central extracellular electron transfer apparatus (*ndh2*::tn) exhibited a defect in colonizing the intestinal lumen and tissue (Fig. 2). To determine if fumarate reduction contributed to this phenotype, we then coinfected mice with the Δ*frdA* strain. In contrast to the other extracellular electron transfer mutants, *L. monocytogenes* that lacked FrdA were recovered from both the gut lumen and gut tissue in approximately equal numbers to wild-type bacteria (Fig. 2). We thus conclude that the extracellular electron transfer apparatus is important for intestinal colonization, but that fumarate is not the biologically relevant electron acceptor in this context.

Gram-Positive Bacteria Possess Evolutionarily Divergent Flavinylated Extracellular Reductases. *L. monocytogenes* FrdA is part of a large and divergent protein superfamily (34). Based on this observation, we reasoned that evolutionarily related but functionally distinct reductases might similarly interact with the Gram-positive extracellular electron transfer apparatus. To identify such proteins, we selected 382 species of Gram-positive bacteria that possess the core extracellular electron transfer genes. Using FrdA reference sequences, we identified homologous proteins from selected genomes and used these to construct a phylogenetic tree. Because we were primarily interested in proteins that could interact with the extracellular electron transfer apparatus, we removed genes that lacked a computationally predicted lipidation site from the dataset. To further facilitate the functional analysis, we identified putative flavinylation sites and active site amino acids within the sequence alignment (*SI Appendix*, Figs. S3 and S4). The resulting phylogenetic tree is defined by three

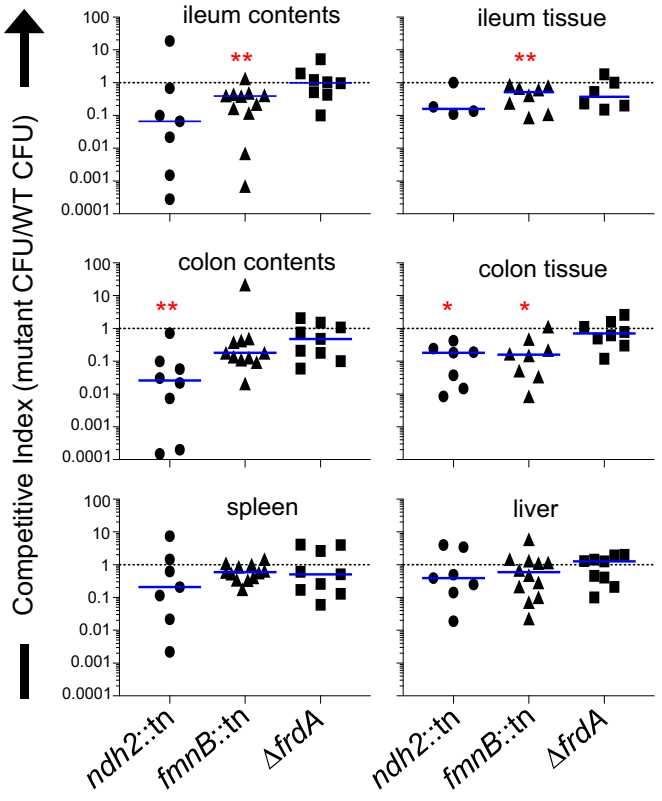

Fig. 2. Extracellular electron transfer genes exhibit a fumarate reductase-independent role in intestinal colonization. Groups of female BALBc/By/J mice were coinfected with a 1:1 mixture of wild-type (DP-L3903) and mutant (*ndh2*::tn, DP-L6614; *fmnB*::tn, DP-L6612; Δ*frdA*, DP-L6757) *L. monocytogenes*. Three days postinfection, the number of CFU for each strain that was either tissue-associated or in the luminal contents of the ileum and colon was determined. Pooled data for mice infected in two separate experiments are shown as competitive indices (# of mutant CFU/# of wild type CFU × 100). Asterisks denote median values (blue lines) that differed significantly from the theoretical ratio of 1.0 (dotted lines) by a Wilcoxon test (*$P < 0.05$; **$P < 0.01$).

major clades (Fig. 3, *SI Appendix*, Fig. S3, and Dataset S1), which are described below.

Clade I contains the most sequences and can be divided into three subclades (Iα, Iβ, and Iγ) (Fig. 3 and *SI Appendix*, Fig. S3). FrdA active site amino acids are strictly conserved in members of clade I (*SI Appendix*, Fig. S4). Consistent with clade I consisting of FrdAs, several species represented in the clade have previously been reported to possess fumarate reductase activity (35–37). The *L. monocytogenes* FrdA is contained within subclade Iα, as are other proteins with a similar flavinylation sequence motif. Members of subclade Iβ retain a flavinylation site, which, compared to Iα sequences, is found in the context of a larger N-terminal domain. Compared to the other subclades, clade Iγ sequences are shorter and lack a flavinylation site. Interestingly, clade Iγ genes are exclusively found in operons that contain a second gene that encodes a small predicted flavinylated lipoprotein. Thus, clade Iγ may reflect a system in which electron transfer and reductase functionalities have been separated into distinct polypeptide chains.

Clade II can be divided into two subclades (IIα and IIβ) (Fig. 3 and *SI Appendix*, Fig. S3). Clade II proteins retain the flavinylation site and possess a subtly different active site than clade I enzymes. An active site histidine that is critical for fumarate reductase activity corresponds to a tyrosine in clade IIα and leucine or methionine in clade IIβ (*SI Appendix*, Fig. S4). This active site signature has previously been associated with the enzyme urocanate reductase, or UrdA, which reduces urocanate (an intermediate in histidine catabolism) to imidazole propionate and can support respiratory growth (Fig. 4*A*) (38). We thus anticipate that clade II contains enzymes with urocanate reductase activities.

Clade III sequences are highly divergent, but almost always retain the N-terminal flavinylation site (Fig. 3 and *SI Appendix*, Fig. S3). Members of the clade exhibit considerable intra- and interclade active site diversity and likely encompass as many as ten distinct enzyme classes (*SI Appendix*, Fig. S4). We identify the single highly represented enzyme family (with 24 sequences) as subclade IIIα. Since the majority of other putative enzyme classes contain <4 representatives, no other subclades have been assigned. Clade III thus likely contains a variety of enzymes with distinct, but presently unidentified substrate specificities.

Flavinylated Urocanate Reductase Exploits a Conserved Extracellular Electron Transfer Mechanism. In order to test whether enzymes from different clades of our phylogenetic tree similarly receive electrons from the extracellular electron transfer apparatus, we asked whether members of clade II possess urocanate reductase activity. To test this, we selected an *Enterococcus rivorum* clade II gene (hereafter called *Er*UrdA) for recombinant expression in *L. monocytogenes* (Fig. 4*B*). Consistent with the hypothesis, we found *Er*UrdA imparted *L. monocytogenes* with urocanate reductase activity (Fig. 4*C*). We further found that *Er*UrdA-mediated urocanate reduction supported anaerobic growth of *L. monocytogenes* on the sugar alcohol xylitol (Fig. 4*D*). These results thus show that a representative member of clade II possesses a urocanate reductase activity that can be functionally reconstituted in *L. monocytogenes*.

We next asked whether extracellular electron transfer was required for *Er*UrdA function. We observed that mutants that prevented posttranslational flavinylation (*fmnB*::tn) or impaired the central extracellular electron transfer apparatus (*ndh2*::tn) lacked *Er*UrdA-mediated urocanate reduction and growth enhancement

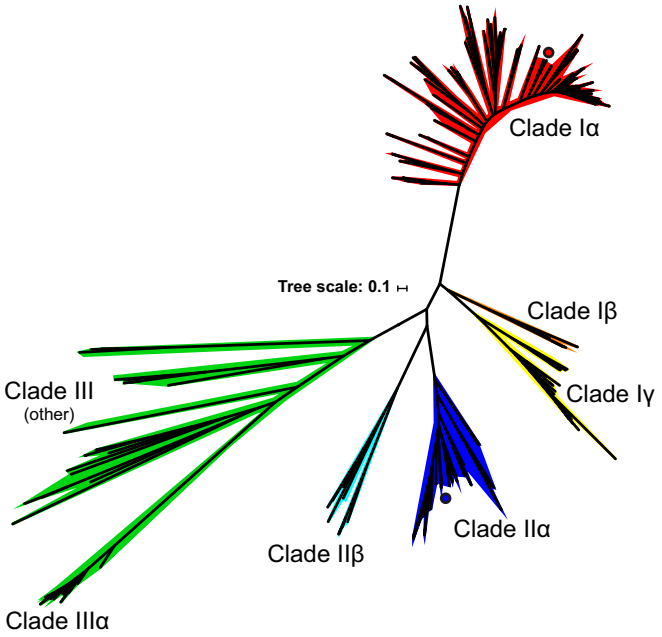

Fig. 3. Phylogenetic tree of flavinylated extracellular reductases (Maximum likelihood tree under an LG+F+I+G4 model of evolution). Scale bar indicates substitutions per site. *L. monocytogenes* FrdA and *Er*UrdA are indicated by a red and blue dot, respectively. A detailed version of the phylogenetic tree is presented in *SI Appendix*, Fig. S3. Dataset S1 in the *SI Appendix* contains the tree with full bootstrap values in Newick format.

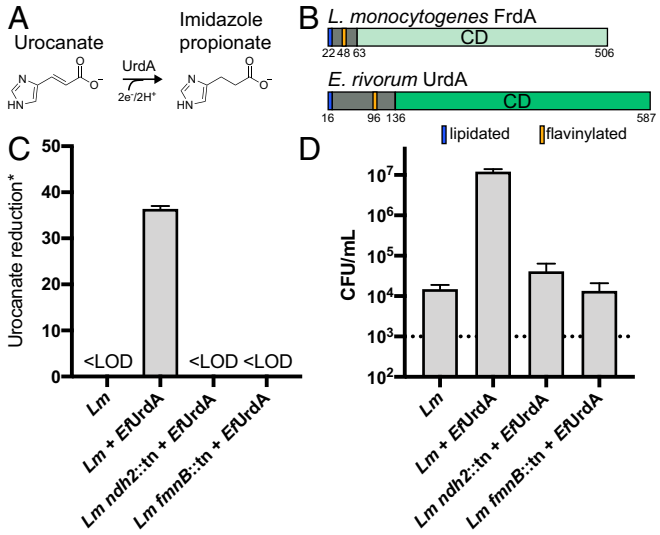

Fig. 4. An extracellular urocanate reductase reconstituted in *L. monocytogenes* requires extracellular electron transfer. (*A*) Reaction catalyzed by urocanate reductase. (*B*) Comparison of domain layout of FrdA from *L. monocytogenes* and UrdA from *E. rivorum*. Catalytic domains (CD) are colored green and the flavinylated N-terminal domain is colored gray. (*C*) Relative imidazole propionate concentration in the spent medium of strains cultivated in medium supplemented with urocanate. Values report the percent imidazole propionate ion counts relative to urocanate plus imidazole propionate ion counts as determined by LC-MS. Below limit of detection (<LOD) indicates conditions in which imidazole propionate was not detected. *L. monocytogenes* strains with *Ef*UrdA and with mutations in the extracellular electron transfer apparatus are indicated. Results (n = 3) are expressed as means and SDs. (*D*) Growth of indicated strains on xylitol/urocanate medium. The CFU/mL after a 48-h incubation are shown, with the dashed line representing the CFU in the inoculum. Results (n = 3) are expressed as means and SDs.

(Fig. 4 *C* and *D*). We thus conclude that urocanate reductases mechanistically parallel fumarate reductases in utilizing a flavinylation site to receive electrons from the extracellular electron transfer system. Furthermore, these results reveal that the electron transfer system is conserved and the reductases sufficiently modular as to permit functional interchangeability of key components between species.

Extracellular Electron Transfer May Represent a Significant Respiratory-Like Activity in Gram-Positive Bacteria. Model extracellular electron transfer organisms, such as the *Shewanella* species, employ a metabolic strategy that is defined by prolific respiratory capabilities (39). By contrast, the types of microbes identified in our studies tend to be more closely associated with fermentative metabolisms. Therefore, to help clarify the significance of extracellular electron transfer in these microbes, we sought to develop a better understanding of their metabolic potential.

We performed a comprehensive analysis of the respiratory capabilities encoded in the 382 genomes identified as possessing components of the extracellular electron transfer system. Initially focusing on previously characterized respiratory systems, we found that 205 of the genomes possess quinol oxidases for aerobic respiration, with 35% of these also possessing nitrate reductases for anaerobic respiration (Dataset S2). No other characterized respiratory systems were identified in these analyses. For many of these organisms, aerobic respiration and nitrate reduction have been reported to depend upon an exogenous source of heme and, thus, even the identified capabilities likely only function in a subset of relevant ecological niches (40). These observations thus suggest that previously characterized respiratory capabilities are limited or nonexistent (no genes were identified in 177 genomes) in microbes that possess the core extracellular electron transfer apparatus.

Next, we analyzed the distribution of the identified flavinylated extracellular reductases across the 382 genomes within our dataset. We found that representatives of these enzymes were present in 172 genomes (Dataset S2). Putative fumarate reductases in clade I of phylogenetic tree were the most common (present in 159 genomes), with clade II putative urocanate reductases also being present in a significant fraction of genomes (42 in total). Many genomes possess multiple flavinylated extracellular reductases, with some having as many as seven from up to four different phylogenetic subclades. These observations thus suggest that extracellular electron transfer capabilities represent a respiratory or respiratory-like functionality comparable to conventional respiratory mechanisms in a subset of Gram-positive bacteria.

Discussion

The studies described here set out to address the ability of *L. monocytogenes* to use respiratory electron acceptors and led to the discovery of a new family of Gram-positive extracellular reductases. These enzymes share an essential ApbE-like post-translational flavin-peptide motif with PplA, the extracellular protein previously implicated in extracellular electron transfer, and depend upon the core extracellular electron transfer apparatus for activity. We propose that this flavin-peptide motif establishes a molecular signature that is recognized by membrane components of the extracellular electron transport chain. This conserved structural signature thus provides a modular mechanism for directing electrons to multiple extracellular proteins with distinct substrate specificities.

These findings extend the role of the ApbE-like flavinylation motif, which increasingly seems to play an important role in various mechanisms of microbial energy metabolism. ApbE-like flavinylation was originally shown to be essential for NQR (NADH-quinone reductase) and RNF (*Rhodobacter* nitrogen fixation) complexes, which drive ion pumps that play a crucial role in the energy metabolism of a number of microbes (30, 41–44). In addition to ApbE-like flavinylation being implicated for the function of other respiratory and nonrespiratory reductases, the prevalence of functionally unassigned ApbE-like enzymes and associated substrate proteins in microbial genomes has been previously noted (31, 32, 45, 46). As such, there likely remains much to be learned about the scope and significance of ApbE-like flavinylation in microbial energy metabolism.

Interestingly, the Gram-positive system characterized here bears some similarities to the periplasmic electron transfer system exemplified by Gram-negative *Shewanella* species. *Shewanella* are mineral-respiring specialists that route extracellular electron transfer activity through the periplasm. *Shewanella* are also unusual in possessing periplasmic fumarate and urocanate reductases. These enzymes resemble the Gram-positive system in requiring extracytoplasmic flavins and in receiving electrons from quinones in the cytoplasmic membrane. Despite these similarities, the *Shewanella* system depends upon a distinct flavin transporter (to provide the noncovalently bound flavin adenine dinucleotide cofactor) and quinol oxidase (47–50). Convergent evolutionary processes thus seem to have resulted in a similar repertoire of extracytoplasmic activities in Gram-negative and Gram-positive lineages.

It is possible that the independent evolution of reductases with extracytoplasmic localization reflects a general advantage of utilizing electron acceptors outside the cytosol. On the other hand, as both Gram-positive and Gram-negative systems rely on elements of an apparatus that can also be used for extracellular iron reduction, the extracytoplasmic localization of reductases may have evolved to exploit features of a preexisting

extracellular electron transport chain. As such, additional cost/benefit analyses will be necessary to clarify the functional significance of extracytoplasmic electron acceptor usage in microbial energy metabolism.

Another set of questions stimulated by this research concerns the substrate utilization of flavinylated extracellular reductases. A number of enzymes identified in this study act on unknown substrates (i.e., clade III of the phylogenetic tree) and thus hint at existence of previously uncharacterized respiratory-like activities. Even in the case of fumarate and urocanate reductases, the ecological context in which their substrates are available at physiologically relevant concentrations remains unclear. Considering the evolutionarily conservation of these enzymes in many Gram-positive genomes, it stands to reason that fumarate and urocanate must be available in some environments.

Interestingly, one environment in which microbes appear to encounter urocanate is within the mammalian gut. A recent report found that patients with type 2 diabetes possess microbiomes with elevated urocanate reductase activity (51). This activity may be linked to the disease, as the product of urocanate reduction, imidazole propionate, was found to influence insulin signaling (51). While the specific microbes responsible for this increase in imidazole propionate remain to be identified, the extracellular urocanate reductases identified here are present in some gut-associated classes of bacteria and thus may contribute to the phenotype.

In conclusion, the results described here establish that a posttranslational flavination motif is used by a family of extracellular reductases to facilitate electron transfer to different extracellular electron acceptors. This system has modular and interchangeable properties that could potentially be exploited for metabolic engineering applications. Finally, the presence of this system in a number of bacteria that exhibit largely fermentative features generates new questions about the basis of metabolism and contributes to a more nuanced understanding of extracellular electron transfer throughout microbial life.

Methods

***L. monocytogenes* Strains and General Growth Conditions.** All strains of *L. monocytogenes* used in this study were derived from the wild-type 10403S (streptomycin-resistant) strain (*SI Appendix*, Table S1) (52). For routine experiments, *L. monocytogenes* cells were grown at 37 °C and spectrophotometrically measured by optical density at a wavelength of 600 nm (OD_{600}). For in vivo infection of mice, strains were grown statically at 30 °C to early stationary phase and aliquots were prepared and frozen until use (53). Filter-sterilized brain-heart infusion medium (Difco) was used for standard *L. monocytogenes* cultivation. An anaerobic chamber (Coy Laboratory Products) with an environment of 2% H_2 balanced in N_2 was used for studies that required anaerobic conditions.

Genetic Manipulation of *L. monocytogenes*. The Δ*frdA* and *frdA* S48A strains were generated using the pKSV7 vector and following a previously described allelic exchange method (54). For complementation, *urdA* and *frdA* were subcloned downstream of the *frdA* promoter into a derivative of the pPL2 vector (55). Complementation with *frdA* and *urdA* was achieved by genomic integration of the pPL2 vector derivatives into the Δ*frdA* strain (55). For introducing transposons that disrupted *fmnB* and *ndh2* into the Δ*frdA* + *urdA* strain, generalized transduction protocols used phage U153, as previously described (56).

Characterization of *L. monocytogenes* Electron Acceptor Usage. To test *L. monocytogenes* electron acceptor usage capabilities, cells were cultivated in a chemically defined medium modified from a previously described formulation (57). This new formulation contained 50 mM xylitol, M9 salts (33.7 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.55 mM NaCl, and 9.35 mM NH_4Cl), minerals (1.7 mM $MgSO_4$, 1 mM $Na_2S_2O_3$, 5 μM $FeCl_3$, 50 μM $MnSO_4$, 1 μM $ZnSO_4$, 10 μM $CaCl_2$, 100 nM $CuSO_4$, 100 nM $CoCl_2$, 100 nM H_3BO_3, 100 nM Na_2MoO_4, and 100 μM sodium citrate), vitamins (2 μM biotin, 1.3 μM riboflavin, 7 μM para-aminobenzoic acid, 20 nM lipoic acid, 8 μM nicotinamide, 4 μM D-pantothenic acid, 5 μM pyridoxal, and 3 μM thiamine), and select amino acids (50 μM each of L-arginine, L-histidine, DL-isoleucine, L-leucine, DL-methionine, L-phenylalanine, L-tryptophan, and DL-valine). For relevant experiments, the medium was supplemented with potential electron acceptors at a 10–50 mM concentration. A preliminary screen revealed that cholate, citrate, dimethyl sulfoxide, malate, nitrite, nitrate, pyruvate, thiosulfate, and trimethylamine *N*-oxide failed enhance *L. monocytogenes* growth. Therefore, subsequent studies exclusively used ferric ammonium citrate, fumarate, and urocanate as electron acceptors. Growth experiments were typically initiated with ∼1000 colony-forming units of *L. monocytogenes*. After incubation in an anaerobic environment for 48–72 h, cultures were serially diluted and plated for the enumeration of colony-forming units.

Fumarate reductase activity was assayed by measuring the concentration of succinate in spent medium from *L. monocytogenes* cultures grown 24 h in brain-heart infusion medium supplemented with 50 mM fumarate. Succinate measurements were made using a commercial colorimetric assay kit (Sigma), following instructions provided by the manufacturer. To assay urocanate reductase activity, *L. monocytogenes* strains were grown anaerobically for 48 h on chemically defined xylitol medium supplemented with 25 mM urocanate. Concentrations of urocanate and imidazole-1-propionate in the spent medium were measured, using a previously described method (51). Briefly, after derivatization with 1-butanol, urocanate and imidazole-1-propionate were characterized by LC-MS.

Assay of FmnB FMN Transferase Activity. Constructs of *fmnB* and *frdA* that truncated the signal peptide and lipidation site were subcloned into the pMCSG58 and pET46-Ek/LIC expression vectors, respectively. The *fmnB* construct encoded residues 37–360 of FmnB and the *frdA* construct encoded residues 31–506 of FrdA. Overexpression and purification of FmnB followed previously described protocols (4), with 6xHis-tag being removed from the *frdA* construct by following the protocol provided by the Enterokinase-His, Bovine (GenScript) manufacturer. To assay FmnB activity, purified FrdA and FmnB were incubated overnight at a 10:1 molar ratio in assay buffer (0.5 M NaCl and 10 mM Tris, pH 8.3) supplemented with 1 mM FAD. Posttranslational modifications of FrdA were determined by mass spectrometric analyses of intact proteins and tryptic peptides, as previously described (4).

Oral *L. monocytogenes* Infection of Mice. Four-week-old female BALBcBy/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME), acclimated to a reversed light cycle in a specific pathogen-free facility for at least 2 wk, and used in experiments at 6–8 wk of age. Mice were fasted overnight on raised wire flooring and then fed a mixture consisting of $\sim 4 \times 10^8$ colony-forming units of wild-type (erm^R DP-L3903) and $\sim 4 \times 10^8$ colony-forming units of mutant *L. monocytogenes* as described previously (58). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Colons and ileums were harvested aseptically, flushed with 8 mL of sterile PBS, and squeezed to collect luminal contents. Flushed tissues were homogenized for 1 min in sterile water using a PowerGen 1000 at 60% power (Fisher). Serial dilutions were prepared for each sample and then double plated on BHI $Strep_{200}$ and BHI $Strep_{200}/Erm_2$ agar plates. The number of mutant *L. monocytogenes* was determined by subtracting the number of erm^R colony-forming units from number of colony-forming units on the BHI/$Strep_{200}$ plates (59).

Bioinformatics Analyses. To identify possible flavinylated extracellular reductases, genomes from species previously identified as possessing extracellular electron transfer genes were selected (4). From these genomes, proteins were predicted using Prodigal (version 2.6.3) (-m -p single options) (60). Protein sequences with homology to the *L. monocytogenes* FrdA were detected using the protein sequence searching software MMseqs2 (version 9f493f538d28b1412a2d124614e9d6ee27a55f45) (-s 7.5 -e 0.001–threads 6–max-sEqs. **5000**–alignment-mode 3–num-iterations 3 -c 0.80 options) (61). Proteins that contained putative lipidation sites were identified by SignalP (version 5.0) (62) and subjected to a multiple alignment and a phylogenetic tree reconstruction. Sequences were aligned using MAFFT (version 7.390) (–auto option) (63). Alignment was further trimmed using Trimal (version 1.4.22) (–gappyout option) (64). Tree reconstruction was performed using IQ-TREE (version 1.6.6), as implemented on the CIPRES web server (65, 66), using ModelFinder (67) to select the best model of evolution, and with 1000 μLtrafast bootstrap (68).

To systematically characterize potential electron acceptor usage capabilities in genomes that contain extracellular electron transfer genes, an HMM database was constructed that contained 12 previously described candidate quinone-dependent reductases (69). The 12 HMMs were constructed based on the sequences from the KEGG database (70). Protein sequences from each genome were functionally annotated based on the accession of their best Hmmsearch match (version 3.1) (E-value cutoff 0.001) (71) in the KEGG

database. The results of this analysis are presented as a summary of the likely presence of reductases within a genome in Dataset S2.

Data availability. Data discussed in the paper are available in Datasets S1 and S2.

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