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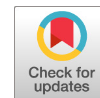

Vibrio fischeri DarR Directs Responses to D-Aspartate and Represents a Group of Similar LysR-Type Transcriptional Regulators

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ABSTRACT Mounting evidence suggests that D-amino acids play previously underappreciated roles in diverse organisms. In bacteria, even D-amino acids that are absent from canonical peptidoglycan (PG) may act as growth substrates, as signals, or in other functions. Given these proposed roles and the ubiquity of D-amino acids, the paucity of known D-amino-acid-responsive transcriptional control mechanisms in bacteria suggests that such regulation awaits discovery. We found that DarR, a LysR-type transcriptional regulator (LTTR), activates transcription in response to D-Asp. The D-Glu auxotrophy of a *Vibrio fischeri murI*::Tn mutant was suppressed, with the wild-type PG structure maintained, by a point mutation in *darR*. This *darR* mutation resulted in the overexpression of an adjacent operon encoding a putative aspartate racemase, RacD, which compensated for the loss of the glutamate racemase encoded by *murI*. Using transcriptional reporters, we found that wild-type DarR activated *racD* transcription in response to exogenous D-Asp but not upon the addition of L-Asp, L-Glu, or D-Glu. A DNA sequence typical of LTTR-binding sites was identified between *darR* and the divergently oriented *racD* operon, and scrambling this sequence eliminated activation of the reporter in response to D-Asp. In several proteobacteria, genes encoding LTTRs similar to DarR are linked to genes with predicted roles in D- and/or L-Asp metabolism. To test the functional similarities in another bacterium, *darR* and *racD* mutants were also generated in *Acinetobacter baylyi*. In *V. fischeri* and *A. baylyi*, growth on D-Asp required the presence of both *darR* and *racD*. Our results suggest that multiple bacteria have the ability to sense and respond to D-Asp.

IMPORTANCE D-Amino acids are prevalent in the environment and are generated by organisms from all domains of life. Although some biological roles for D-amino acids are understood, in other cases, their functions remain uncertain. Given the ubiquity of D-amino acids, it seems likely that bacteria will initiate transcriptional responses to them. Elucidating D-amino acid-responsive regulators along with the genes they control will help uncover bacterial uses of D-amino acids. Here, we report the discovery of DarR, a novel LTTR in *V. fischeri* that mediates a transcriptional response to environmental D-Asp and underpins the catabolism of D-Asp. DarR represents the founding member of a group of bacterial homologs that we hypothesize control aspects of aspartate metabolism in response to D-Asp and/or to D-Asp-containing peptides.

KEYWORDS Aliivibrio, *Photobacterium*, signaling, peptidoglycan, HypT, LTTR

There is a growing interest in the metabolism and function of D-amino acids, which despite being less common than their proteinogenic L-enantiomeric counterparts are widely distributed in the biosphere. D-Amino acids can form spontaneously from

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L-amino acids (1, 2), and in cells, they are often generated enzymatically through the activities of amino acid racemases, which are found in all domains of life and interconvert the D- and L-forms of amino acids (3). In bacteria, there are long-established roles for D-Glu and D-Ala, along with their respective racemases, in generating the conserved canonical structure of the peptidoglycan (PG) cell wall. In addition, D-Glu is found in *Bacillus anthracis* capsule material (4). However, bacteria also encounter and produce a variety of other less familiar D-amino acids with functions that are not well characterized.

The biological significance of these D-amino acids for bacteria is an active area of research. D-Amino acids encountered by bacteria could serve as carbon and/or nitrogen sources (5–8), and in some cases, their presence may serve as cues indicative of particular environments (9). In addition to D-amino acids derived from PG remnants, other D-amino acids may be encountered by bacteria in plant or animal tissues, in soils, or in aquatic environments (5–8, 10, 11). D-Amino acids are also produced by bacteria for various uses (12, 13). In addition to the D-Ala and D-Glu found in PG, bacteria incorporate D-amino acids into antibiotics, surfactants, and noncanonical PG (14–16). Moreover, D-amino acids may be used as signals or as structural components in developmental processes, including spore germination (17–19), growth-phase-dependent cell wall remodeling (20), and biofilm development, although the role in biofilm development is controversial (21–25). Genes predicted to encode amino acid racemases are common in bacterial genomes, yet whether these enzymes primarily serve to generate or to catabolize D-amino acids remains unclear given that racemases typically interconvert D- and L-forms of their substrates.

Whether produced as signals or scavenged as growth substrates, D-amino acids might be expected to elicit transcriptional responses; however, examples of D-amino-acid-responsive transcriptional regulators are rare. One such example is the LysR-type transcriptional regulator (LTTR) DsdC from *Escherichia coli*. DsdC responds to D-Ser and activates the transcription of genes needed to metabolize D-Ser (26), a compound that *E. coli* may encounter in human urine (27). Another LTTR responsive to a D-amino acid is DguR in *Pseudomonas aeruginosa* PAO1. DguR directs the catabolism of D-Glu in response to D-Glu (28) and may help *P. aeruginosa* use PG fragments in the environment. Despite these examples, transcriptional responses to important and widespread D-amino acids are generally unknown.

One D-amino acid of interest is D-Asp. In addition to being found in the environment, endogenous D-Asp forms in vertebrate and invertebrate tissues. Regional and temporal patterns of D-Asp accumulation suggest that it plays important physiological roles in the neuroendocrine systems of phylogenetically distant organisms, including amphibians, chickens, rodents, and humans (29–31). D-Asp is also found in various tissues of marine invertebrates (32–34). Interestingly, D-Asp and iso-D-Asp are generated as a consequence of repairing iso-Asp in aging proteins, and D-Asp or iso-D-Asp may in turn serve as signals or as markers of protein damage (35). Although several bacterial genomes include annotated aspartate racemase genes, the roles and regulation of such racemases remain unknown.

In this study, we describe a novel LTTR identified in the marine bacterium *V. fischeri*. This regulator was designated DarR, for D-aspartate-responsive regulator. Bioinformatic analyses revealed additional bacteria that have *darR* homologs genetically linked to genes with predicted functions in D-amino acid and/or aspartate metabolism. We propose that *V. fischeri* DarR represents a larger family of D-amino-acid-responsive LTTRs with varied ecological roles.

RESULTS

A mutation in *darR* suppresses the D-Glu auxotrophy of a *murI* mutant. In a recent search for transposon insertions in conditionally essential genes, we recovered the *murI*::miniTn*5-erm* mutant AN3, which requires D-Glu supplementation for growth (36). The D-Glu auxotrophy of this *murI* mutant is consistent with the established function of MurI in other bacteria as a glutamate racemase required to provide the

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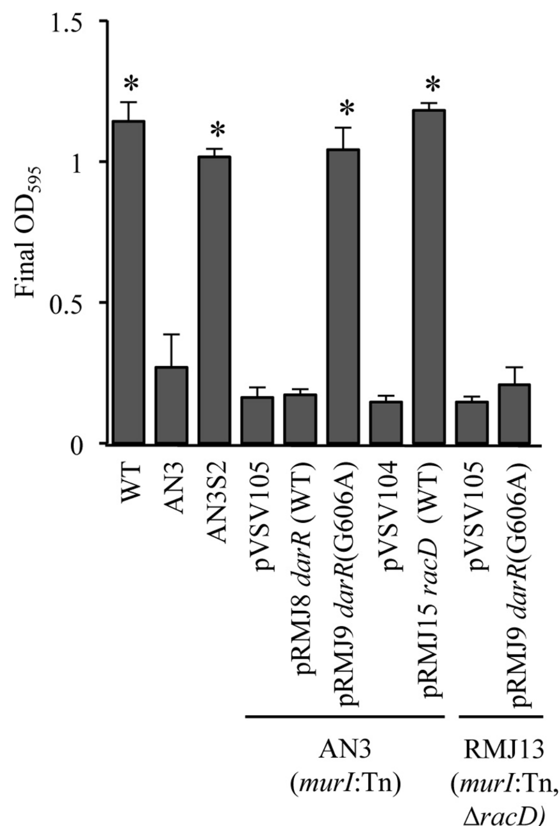

FIG 1 Restoration of prototrophy to the D-Glu auxotroph AN3 by *darR*(G606A) or by multicopy *racD* in *trans*. Strains were subcultured (1:1,000) from broth cultures at an OD_{595} of 0.6, grown for 14 h in LBS, and the final OD_{595} was recorded. Asterisks indicate a significant difference relative to AN3 (*murI*::Tn), as determined by Student's *t* test ($P < 0.01$). Error bars indicate the standard error ($n = 3$). Data from one representative experiment among three are shown. WT, wild type.

D-Glu moiety in PG. We selected spontaneous suppressors of D-Glu auxotrophy in AN3 by plating on lysogeny broth salt (LBS) medium without D-Glu. The frequency of suppression was approximately 10^{-7}, based on dilution plating in parallel on LBS supplemented with D-Glu (data not shown). We analyzed three suppressor mutants, but these may have been siblings, as they were indistinguishable from each other in the amino acid content of their PG, in growth assays, and in whole-genome resequencing analyses. We therefore report the characterization of the mutant strain AN3S2 as a representative of these suppressor mutants. The growth defect of parent strain AN3 (*murI*::miniTn*5-erm*) in the absence of D-Glu was significantly reversed in the suppressor AN3S2 (Fig. 1). However, the PG of AN3S2 was indistinguishable from that of the wild type in its amino acid and muropeptide content based on high-performance liquid chromatography (HPLC) analysis (data not shown). These biochemical PG analyses cannot rule out the unprecedented substitution of L-Glu for D-Glu, but they more likely indicate a restoration of wild-type PG.

The genome sequence of AN3S2 revealed a point mutation in gene VF_1545 (*darR*), which encodes a previously uncharacterized LTTR (Fig. 2A). The mutation (G606A) results in a single amino acid replacement (M202I) in the putative effector-binding domain (EBD) of the protein (Fig. 2A). To determine whether the point mutation found in *darR* was responsible for the suppression of D-Glu auxotrophy, we tested whether this allele restored prototrophy to strain AN3 in *trans*. When added on a plasmid, the mutant *darR* encoding DarR(M202I) reversed the D-Glu auxotrophy of AN3, whereas a plasmid carrying the wild-type *darR* or an empty-vector control did not (Fig. 1). These results suggest that the DarR variant accounts for the suppression phenotype.

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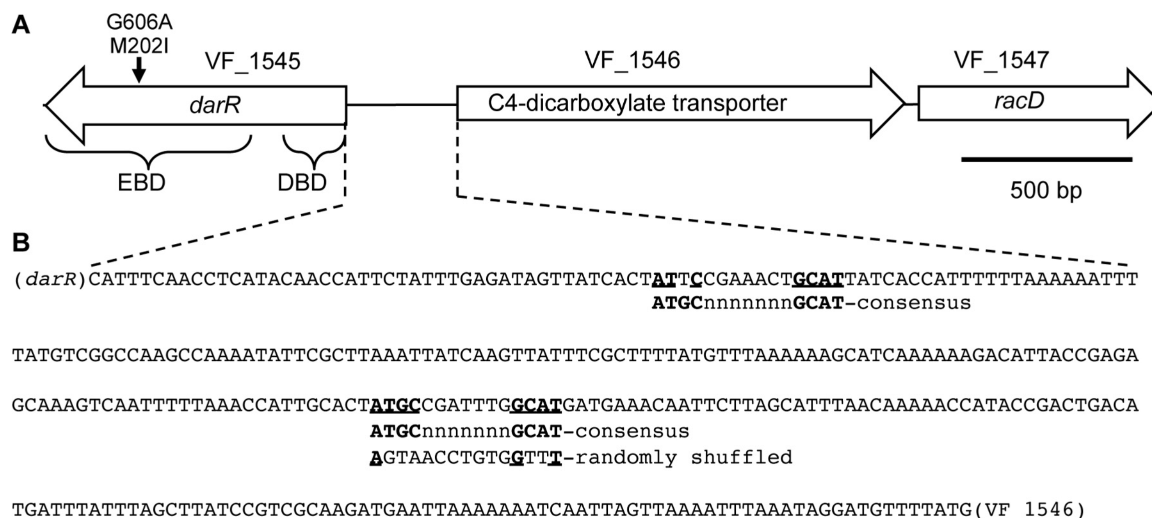

FIG 2 The *V. fischeri darR* locus. (A) Horizontal arrows represent ORFs VF_1545 (*darR*), VF_1546, and VF_1547 from *V. fischeri* ES114 chromosome 1. Shown on *darR* is the site of the G606A mutation (causing M202I amino acid replacement in DarR) in the suppressor mutant AN3S2, the regions encoding the effector-binding domain (EBD), and the DNA-binding domain (DBD) of the LTTR. (B) Intergenic region between and including the ATG start codons for *darR* (reverse strand) and VF_1546. Two putative DarR-binding sites are shown, one a perfect match to the proposed consensus and another with a 1-bp mismatch. The sequence used to randomly shuffle the exact consensus site in control experiments is indicated.

Overexpression of a putative Asp racemase suppresses the D-Glu auxotrophy of the *murI* mutant. As illustrated in Fig. 2A, the *darR* gene is divergently oriented adjacent to a predicted two-gene operon encoding a C_4-dicarboxylic acid transporter (VF_1546) and an Asp racemase (*racD*; VF_1547). This genetic arrangement, characteristic of genes that encode LTTRs, suggests that DarR controls the transcription of *racD*. Based on the similar chemical structures of Glu and Asp, we hypothesize that RacD is an Asp racemase that can act on Glu as a substrate, albeit poorly, such that increased production of RacD compensates for a loss of the *murI*-encoded Glu racemase. We therefore predicted that DarR(M202I) activates higher-than-wild-type levels of *racD* transcription. Three lines of evidence support this model. First, the suppressive effect of the *darR* mutation requires a functional *racD*, as evidenced by the failure of the mutant *darR* allele (on pRMJ9) to restore prototrophy to a *murI* mutant when *racD* is deleted (compare effects of pRMJ9 on AN3 and RMJ13 in Fig. 1). Second, the overexpression of *racD* from a multicopy plasmid was sufficient to restore D-Glu prototrophy to AN3 (Fig. 1). Third, a P_{racD}-*gfp* reporter showed increased expression in the mutant encoding DarR(M202I) but decreased expression in a Δ*darR* mutant (Fig. 3). This P_{racD}-*gfp* reporter (pRMJ11) was constructed by cloning the intergenic region between *darR* and open reading frame (ORF) VF_1546 (Fig. 2) upstream of *gfp* in a reporter plasmid. There are only 41 bp between VF_1546 and *racD* (VF_1547), and the DOOR operon prediction database predicts that these genes are cotranscribed (37), so it seems likely that this cloned fragment includes a transcriptional promoter for *racD*. Together, these data suggest that *racD* expression is responsible for the suppression of D-Glu auxotrophy and that DarR regulates the expression of the *racD* operon.

Dose-dependent stimulatory effect of exogenous D-Asp on a P_{racD}-*gfp* reporter. We hypothesized that DarR regulates the transcription of *racD*, encoding an Asp racemase, in response to L- or D-Asp. Consistent with DarR directing a regulatory response to D-Asp, the P_{racD}-*gfp* reporter displayed dose-dependent expression with increasing D-Asp, showing a significant response ($P < 0.01$) to as little as 125 μM exogenous D-Asp (Fig. 4). This response is *darR* dependent, as a Δ*darR* strain showed no response to D-Asp (Fig. 3). Moreover, the expression of this reporter was not altered detectably by the addition of 16 mM L-Asp, D-Glu, or L-Glu (Fig. 4), indicating a degree of specificity for D-Asp.

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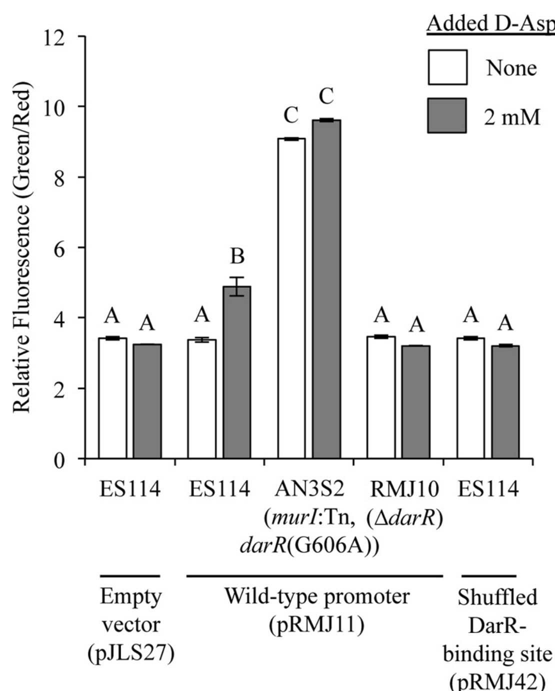

FIG 3 Effect of *darR* and D-Asp on expression of P_{racD}-*gfp* reporter. Relative P_{racD}-*gfp* reporter activity is presented as GFP expression (green fluorescence) normalized to constitutive mCherry (red fluorescence) expression for strains grown in LBS (None) or LBS supplemented with 2 mM D-Asp. Strains carry either a reporter with the wild-type promoter sequence (pRMJ11) or a version where a putative DarR-binding site has been randomly shuffled (Fig. 1B) in pRMJ42. Values with the same letter are not statistically significantly different ($P > 0.05$), whereas different letters indicate significant differences ($P < 0.01$), based on a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test. Error bars indicate standard error ($n = 3$). Data from one representative experiment among three are shown.

Evidence of autorepression by DarR. In addition to regulating other nearby genes, LTTRs often repress their own expression, even in the absence of an activating effector, to maintain regulator homeostasis (38). To determine the effect of DarR on the expression of its own gene, we constructed a P_{darR}-*gfp* reporter (pRMJ10). This P_{darR}-*gfp* reporter showed a small but significant ($P < 0.01$) increase in expression in a Δ*darR* background, consistent with the DarR protein repressing *darR* transcription (Fig. 5). In the suppressor strain AN3S2, which encodes DarR(M202I), P_{darR}-*gfp* expression drops significantly ($P < 0.01$) below wild-type levels (Fig. 5). Additional studies of this P_{darR}-*gfp* reporter failed to show a consistent response to D-Asp, L-Asp, D-Glu, or L-Glu in strains encoding DarR, DarR(M202I), or no DarR (data not shown). Thus, *darR* appears to mediate the regulation of the P_{darR}-*gfp*, without the D-Asp dependence observed with the P_{racD}-*gfp* reporter (Fig. 3 and 4).

DarR-like regulators are encoded in similar genetic contexts in diverse proteobacteria. We searched sequence databases for DarR homologs and found several genes encoding putative LTTRs with more than 25% overall identity to DarR. The genetic contexts of these genes often suggested functional connections to Asp metabolism and/or racemase activity. Specifically, *darR*-like genes were often linked to genes predicted to encode one of the following five types of products: (i) aspartate racemase, (ii) isoaspartyl dipeptidase, (iii) aspartate-ammonia lyase, (iv) mandelate racemase, or (v) GltI, which is involved in Glu/Asp periplasmic binding and uptake. Out of 155 *darR*-like homologs examined, 123 (79%) homologs were within a 16-kbp window of one or more genes annotated in these five categories. To put this observation in perspective, we examined how often homologs of two other LTTRs from *V. fischeri* (VF_1974 and VF_2606) fell within a 16-kbp window of the same five gene groups listed above. A similar genetic proximity occurred in 0% to 4% of 100 genomes examined, respectively. Potential aspartate transporters are also common in the regions

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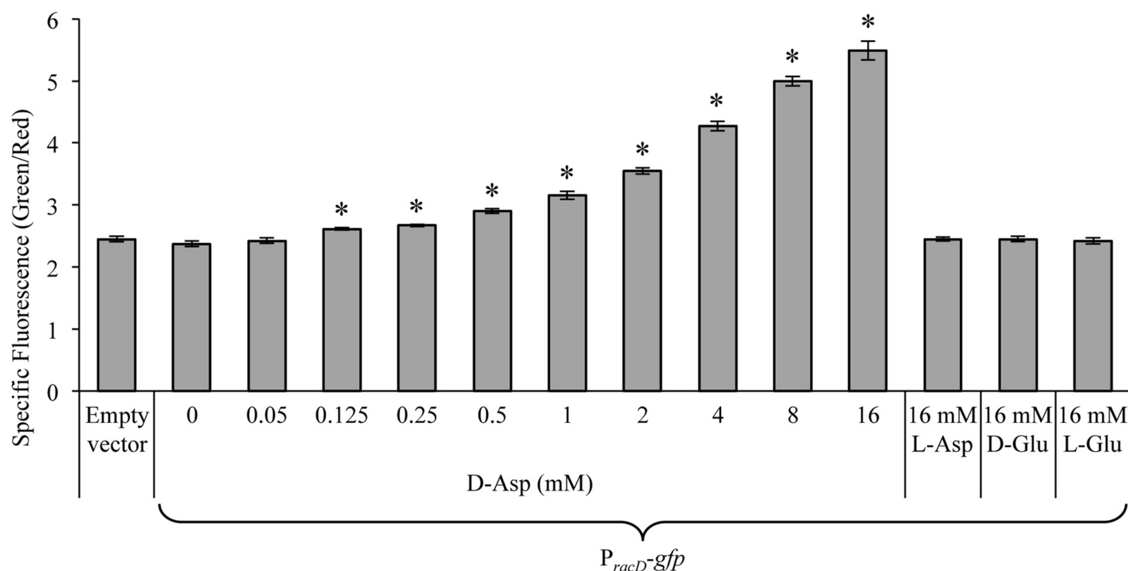

FIG 4 Dose dependence and specificity of P_{racD}-*gfp* reporter response to D-Asp. P_{racD}-*gfp* reporter activity is presented as GFP expression (green fluorescence) normalized to constitutive mCherry expression (red fluorescence) for wild-type strain ES114 carrying the P_{racD}-*gfp* reporter (pRMJ11) or the empty-vector control (pJLS27) grown in LBS or LBS supplemented with the indicated concentration of specific amino acid (D-Asp, L-Asp, D-Glu, or L-Glu). Asterisks indicate significantly more expression relative to the empty-vector control, as determined by one-tailed Student's *t* test ($P < 0.01$). Error bars indicate the standard error ($n = 6$).

near *darR*-like genes, including the predicted C_4-dicarboxylic acid transporter (VF_1546) adjacent to *darR* mentioned above. Figure 6 shows a representative subset of these common genomic arrangements. Notably, some bacteria have more than one *darR*-like gene, including *V. fischeri* ES114, which has a *darR* paralog adjacent to an isoaspartyl dipeptidase gene.

***darR* and *racD* are required for catabolism of D-Asp.** A D-Asp-responsive regulator activating the expression of Asp racemase might allow bacteria to recognize environmental D-Asp and convert it to L-Asp, thereby enabling its catabolism. We tested this possibility using both *V. fischeri* ES114 and another bacterium with *darR* linked to *racD*, *Acinetobacter baylyi* ADP1 (Fig. 6). In addition to *darR* and *racD* being genetically linked in each of these bacteria, homology searches indicate that these genes are reciprocal best matches between the ES114 and ADP1 genomes, suggesting that they are orthologs (39). *V. fischeri* ES114 can use L- or D-Asp as a nitrogen source; however, the loss of *racD* or *darR* reduces or eliminates the ability to grow using D-Asp (Fig. 7A). This effect can be reversed by genetic complementation in *trans* with *racD* or *darR*. Similarly, *A. baylyi* ADP1 can grow on L- or D-Asp as a sole source of carbon. However, the ability to grow on D-Asp is lost in *racD* and *darR* mutants (Fig. 7C). Thus, at least in these two bacteria, *darR* and *racD* are both required for D-Asp catabolism.

Identification of a putative DarR-binding site in the intergenic region between *darR* and the *racD* operon. We used the analysis of *darR*-like homologs described above (Fig. 6) as the basis of a search for a DarR-binding site (see Materials and Methods). In short, we aligned putative promoter sequences upstream from genes we speculated might be regulated by DarR homologs and searched these sequences for a conserved sequence with the canonical 5′-T-N_{11}-A-3′ motif and dyad symmetry typical of LTTR-binding sites (40). In this way, we identified a putative DarR-binding site, 5′-ATGC-N_7-GCAT-3′. The same sequence was independently predicted as an LTTR-binding site upstream of an Asp-ammonia lyase gene in *Acinetobacter baylyi* ADP1 by Craven and colleagues (41). The LTTR predicted to bind this site is encoded by the *darR*-like gene depicted for *A. baylyi* in Fig. 6C, further implicating this sequence as the recognition site for DarR. There is one perfect match to this putative DarR-binding site upstream of VF_1546, and another site with a 1-bp mismatch closer to *darR* (Fig. 2B). Allowing for a 1-bp mismatch from the putative DarR-binding site, there are 2,076

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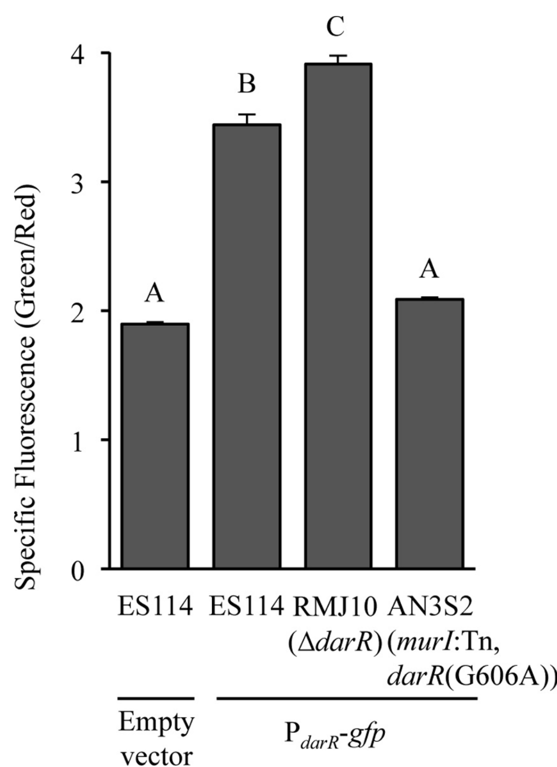

FIG 5 Evidence of DarR autorepression. GFP expression (green fluorescence) from the P_{darR}-*gfp* reporter on pRMJ10 normalized to constitutive mCherry expression (red fluorescence) is shown when the reporter is carried by ES114 (wild type), RMJ10 (Δ*darR* mutant), or AN3S2 (*murI*::Tn *darR*[G606A]). Cultures were grown in LBS, and the promoterless parent vector pJLS27 (indicated as "empty vector") in wild type is shown for comparison. Values with the same letter are not statistically significantly different ($P > 0.05$), whereas different letters indicate significant differences ($P < 0.01$), based on a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test. Error bars indicate the standard error ($n = 3$).

potential binding sites within the *V. fischeri* ES114 genome (see the supplemental material); however, there are only five occurrences of two such sites in the same intergenic region, including the sequence between *darR* and VF_1546. Similarly, only eight out of 90 perfect matches to the DarR-binding sequence fall in intergenic regions (see the supplemental material). Randomly shuffling the putative DarR target site to 5′-AGTAACCTGTGGTTT-3′ eliminated D-Asp-responsive activity in the P_{racD}-*gfp* reporter (Fig. 3).

DISCUSSION

D-Amino acids are widespread in nature, and there is growing appreciation of the roles they play in organisms, yet few regulatory responses to D-amino acids have been described. Here, we report the discovery of DarR, which directs transcriptional responses to D-Asp (e.g., Fig. 3 and 4), and we show that DarR-like homologs are encoded in many bacterial genomes (Fig. 6). Three lines of evidence suggest the functional similarity of these regulators. First, most *darR*-like homologs are oriented near genes predicted to be involved in Asp metabolism. Second, functional similarity is evident in the observation that *darR* mutants of *V. fischeri* and *A. baylyi* were defective in using D-Asp to support growth (Fig. 7). Finally, a putative DarR-binding motif of 5′-ATGC-N_7-GCAT-3′ was identified here in *V. fischeri* and previously (and independently) for a DarR-like homolog in *A. baylyi* ADP1 (41). In *V. fischeri*, this site is required for DarR- and D-Asp-dependent activation of a *racD* reporter (Fig. 3). We therefore posit that *V. fischeri* DarR represents a larger subgroup of LTTRs that warrant further investigation.

Role of the *darR-racD* locus in *V. fischeri*. The manner in which *darR* was discovered suggested that this locus played a role in D-amino acid metabolism. We had

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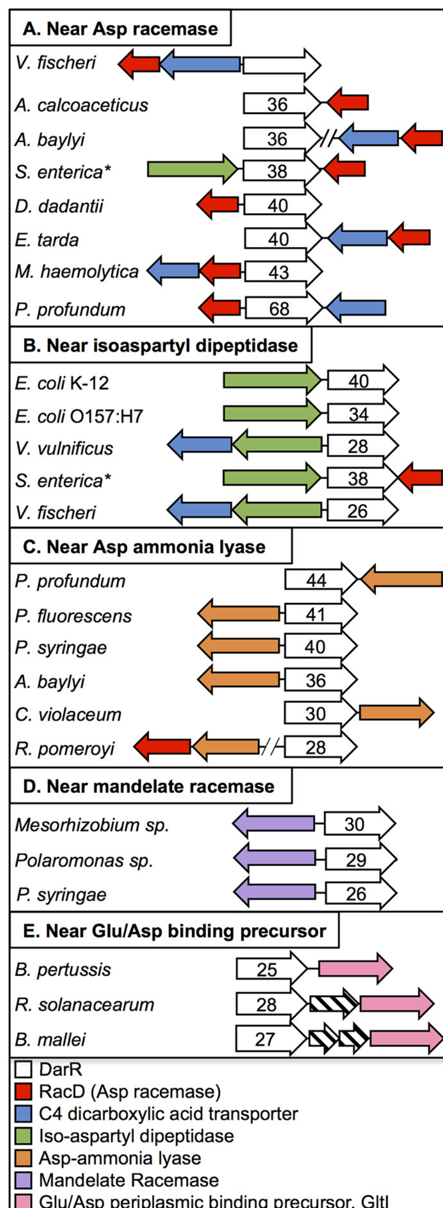

FIG 6 Synteny of *darR*-like homologs in diverse proteobacteria. White arrows represent *darR* homologs, with numbers indicating the percent identity to the DarR sequence from ES114. Homologs are grouped by their proximity to genes annotated as encoding aspartate racemase (RacD) (A), isoaspartyl dipeptidase (B), aspartate-ammonia lysase (C), mandelate racemase (D), or glutamate/aspartate periplasmic binding precursor, GltI (E). Arrows with same color represent genes with the same functional annotation (see color legend at bottom) and striped arrows indicate ORFs that do not fall into any of the groups described above. Asterisks indicate that the same DarR homolog in *S. enterica* is listed in panels A and B, because it is flanked by both Asp racemase and isoaspartyl dipeptidase gene homologs.

attempted to alter the PG of *V. fischeri* by selecting spontaneous suppressors of D-Glu auxotrophy in a *murI* mutant; however, rather than replacing the D-Glu in their PG, the suppressors apparently used an alternative route to synthesize D-Glu-containing wild-type PG. The most parsimonious explanation for this observation consistent with our data is that a mutation in *darR* led to the overexpression of RacD, an enzyme that has some ability to interconvert L- and D-Glu and thereby substitute for MurI. However, *racD* is predicted to encode an aspartate (not glutamate) racemase, and several lines of evidence suggest the locus evolved for aspartate rather than glutamate metabolism. This evidence includes the requirement of *racD* for growth on D-Asp, the requirement

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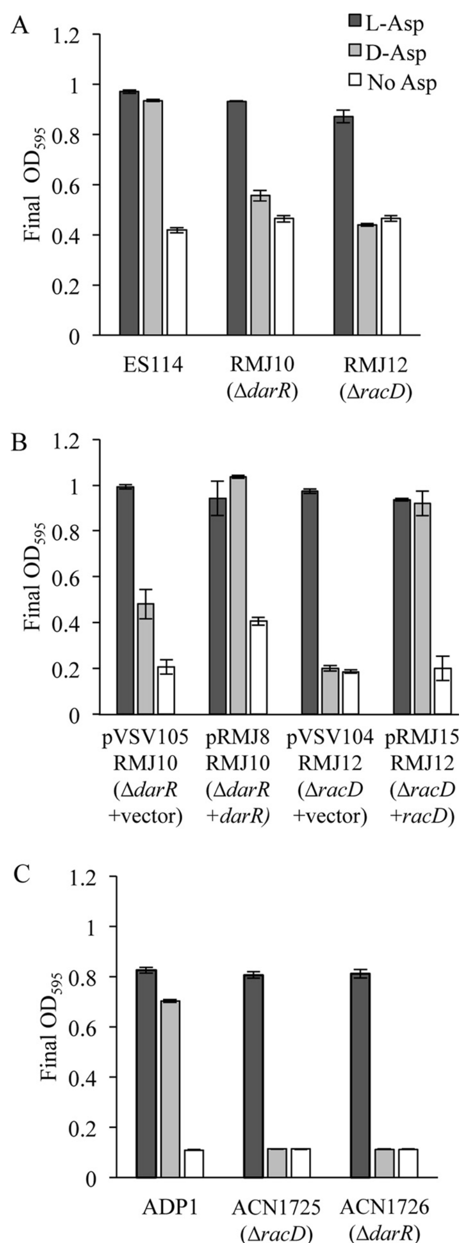

FIG 7 Requirement of *racD* and *darR* for growth on D-Asp. Shown are final OD_{595} readings for *V. fischeri* (A and B) and *A. baylyi* (C) cultures grown with 20 mM L-Asp, D-Asp, or no added aspartate. For *V. fischeri* cultures, Asp was used as an additional nitrogen source with 1 g/liter Casamino Acids in FMM, and 40 mM glycerol was provided as a carbon source. For *A. baylyi*, Asp was used as the carbon source, and 15 mM ammonium chloride was added as a nitrogen source. Cultures were grown with shaking for 24 h before reading the final OD_{595}. Error bars indicate the standard error ($n = 3$).

of D-Asp (but not D-Glu) for induction of the P_{racD}-*gfp* reporter, and the observation that the gene upstream of *racD* encodes a putative C_4-dicarboxylic acid transporter (VF_1546), consistent with transport of D-Asp but not D-Glu. We have shown that DarR mediates the transcriptional activation of this transporter gene and *racD* in response to environmental D-Asp, and we can imagine at least two possible roles for this two-gene operon.

One possibility is that *V. fischeri* uses this locus to catabolize the D-Asp it encounters in the marine environment. This potential function is consistent with the observation that *darR* and *racD* are required for *V. fischeri* to grow on D-Asp as a source of nitrogen (Fig. 7), although *V. fischeri* cannot grow on D- or L-Asp as a sole source of carbon (data

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not shown). D-Asp has been found at levels in the hundred-nanomolar range in marine microcosms (5) and at around 10 μmol/g in specific marine animal tissues (32–34). Although these concentrations are below the threshold for DarR-mediated induction shown in Fig. 4, we have seen induction in some experiments at such D-Asp levels (data not shown), and D-Asp concentrations may be higher in local microenvironments. Preliminary experiments showed no evidence that the P_{racD}-*gfp* reporter was induced during the colonization of the symbiotic host of *V. fischeri*, the bobtail squid *Euprymna scolopes* (data not shown), but this possibility merits further investigation.

Alternatively, or in addition, to D-Asp catabolism, bacteria, including *V. fischeri*, may generate D-Asp as a signaling molecule, with a response directed by DarR. If RacD activity resulted in a net production of D-Asp, this setup would resemble the positive-feedback signaling circuit composed of *V. fischeri* LuxI and LuxR, which generate and respond to *N*-3-oxo-hexanoyl-homoserine lactone, respectively (42). One observation inconsistent with a role in signaling is that the suppressor mutant with a hyperactive DarR, which appears to increase RacD activity, did not release enough D-Asp to trigger a response in nearby cells (data not shown). Still, we cannot rule out signaling or other possible functions for the locus.

Future work should further help test possible roles for the *darR-racD* locus. Investigating the enzyme kinetics and specificity of RacD would not only test whether it is capable of generating D-Glu, as we hypothesize, but it might help resolve its function for *V. fischeri*. An Asp/Glu racemase in *E. coli* was described that favors the conversion of L- to D-Glu (43), and such unidirectional (or biased) interconversion could speak to the enzyme's role. For example, bias in the D-to-L-Asp direction would be consistent with catabolism of environmental D-Asp and its incorporation into central metabolism (e.g., into proteins) but would not support the idea that this operon generates D-Asp as a signaling molecule. Defining the DarR regulon could also elucidate its function. For example, if D-Asp generated by RacD underlies a signaling mechanism, presumably, DarR will control other genes, which could be assessed in global transcriptomic experiments.

Role of DarR-like homologs in other bacteria. Figure 6 illustrates some of the many *darR* homologs found in bacteria, and the genomic contexts of these genes reveal patterns that may reflect a conserved function(s). Genes encoding DarR homologs tend to be found at loci that also encode Asp racemases, isoaspartyl dipeptidases, Asp-ammonia lyases, mandelic acid racemases, or the periplasmic Glu/Asp binding precursor GltI. Thus, as noted above, most DarR homologs are encoded near genes involved in some way with Asp metabolism. We speculate that many of these gene clusters are transcribed in response to D-Asp encountered in the environment and in some way direct its metabolism.

We also hypothesize that the DarR-like regulators encoded near isoaspartyl dipeptidase genes respond to D-Asp linked to other amino acids rather than to free D-Asp. These DarR homologs are widespread and even include one DarR paralog in *V. fischeri* (Fig. 6B). Given the function of isoaspartyl dipeptidase, we speculate that these LTTRs respond to D-iso-Asp in peptides originating from damaged proteins. Isoaspartate residues are created in aging proteins when Asp residues become isomerized. Isomerization, deamidation, and racemization of Asp and Asn make up a large portion of the damage caused when proteins age (44), with isomerization being the most common form of damage and leading to the generation of iso-Asp residues (45). These damaged residues are resolved to either D- or L-Asp through a succinimide intermediate. The accumulation of iso-Asp residues is countered through the activity of protein L-isoaspartyl *O*-methyltransferase (PIMT), which is found in both eukaryotes and bacteria (46). PIMT is responsible for protein damage repair and contributes to recovery from long-term stationary phase (47). In *Salmonella enterica* serovar Typhimurium, PIMT is important for survival under oxidative stress conditions, like those found upon infection of macrophages (48). Through PIMT activity, methylation of the iso-Asp residue favors regeneration of the succinimide intermediate, thereby favoring resolution to L- or D-Asp. Thus, this damage

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and repair process can ultimately result in the acquisition of D-Asp or D-iso-Asp residues in proteins. Isoaspartyl dipeptidases can cleave linkages between iso-Asp and adjacent amino acids when these damaged proteins are turned over. We speculate that the DarR homologs encoded near isoaspartyl dipeptidase genes may respond to D-iso-Asp-containing dipeptides and regulate their turnover and recycling by the cell.

Interestingly, one DarR homolog encoded near an isoaspartyl dipeptidase gene has been characterized, specifically HypT in *E. coli*. This LTTR mediates a transcriptional response to the reactive oxygen species hypochlorite (49), and it is activated by methionine oxidation (50). Although hypochlorite may directly induce the regulatory activity of HypT, it is tempting to speculate that hypochlorite also results in protein damage and accumulation of D-iso-Asp-containing dipeptides, which might act as the effector or coeffector. Although HypT was previously identified as an ortholog of DarR, it is more likely an ortholog of DarR's paralog (VF_1510), as they share the genetic context of proximity to an isoaspartyl dipeptidase gene (Fig. 6B). Further study is needed to determine if VF_1510 in *V. fischeri* responds similarly to hypochlorous acid (HOCl), which is among the reactive oxygen species *V. fischeri* encounters when it infects the light organ (51, 52).

Broader view of D-amino acids. Our unforeseen discovery of a D-Asp-responsive regulator in *V. fischeri* has opened a window on a number of similar bacterial genes that may likewise direct responses to D-Asp, and it suggests that transcriptional responses to other D-amino acids may exist but remain unknown. D-Amino acids can be formed either through the activity of dedicated racemase enzymes or through unavoidable spontaneous chemical reactions, and they may serve bacteria either as signals or as carbon sources to be scavenged. The functional roles of D-amino acids, the transcriptional responses to them, and how they relate to bacterial ecology should be interesting topics for future research.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1. When added to LB medium (53) for the selection of *E. coli*, chloramphenicol (Cam) and kanamycin (Kan) were used at concentrations 20 and 40 $\mu g \cdot ml^{-1}$, respectively. For selection of *E. coli* with erythromycin (Erm), 150 $\mu g \cdot ml^{-1}$ was added to brain heart infusion (BHI) medium (Difco, Sparks, MD). *A. baylyi* strains were grown in minimal medium with 20 mM pyruvate, 20 mM L-aspartate, or 20 mM D-aspartate added as the carbon source (54). When added for the selection of *A. baylyi*, Kan was added at a concentration of 25 $\mu g \cdot ml^{-1}$. Unless otherwise noted, *E. coli* and *A. baylyi* were incubated at 37°C. *V. fischeri* was grown at 28°C in LBS medium (55) or *Fusarium* minimal medium (FMM) (56), with Asp replacing NH_4Cl. When added to LBS for the selection of *V. fischeri*, Cam, Kan, and Erm were used at concentrations of 2, 100, and 5 $\mu g \cdot ml^{-1}$, respectively. D-Glu was added at a final concentration of 400 $\mu g \cdot ml^{-1}$ for D-Glu auxotrophs, unless otherwise indicated. Agar was added to a final concentration of 1.5% for solid media.

Molecular genetics and sequence analysis. Plasmids (Table 1) were constructed using standard genetic techniques. Oligonucleotides used for PCR and cloning are listed in Table 1 and were synthesized by Integrated DNA Technologies (Coralville, IA). DNA ligase and restriction enzymes were obtained from New England BioLabs (Beverly, MA). PCR was conducted with Phusion DNA polymerase, except in the production of biotinylated probes, which were generated using *Taq* polymerase (New England BioLabs). Plasmids used for cloning were isolated with the Zymo plasmid miniprep kit (Zymo Research, Irvine, CA). DNA was repurified between cloning steps with the DNA Clean and Concentrator-5 kit (Zymo Research). Cloned PCR products were sequenced at the University of Michigan DNA Sequencing Core Facility, except *A. baylyi* alleles, which were sequenced by GeneWiz (South Plainfield, NJ). Sequences were analyzed using Geneious version 8.1 (57). Genome resequencing was performed as described previously (58). The resulting assemblies were analyzed for single-nucleotide polymorphisms (SNPs) using Geneious version 8.1 (57).

Plasmids were generated in *E. coli* and conjugated into *V. fischeri* through triparental mating using the helper plasmid pEVS104 (59) in strain CC118λ*pir* (60). Transcriptional reporters pRMJ10 (P_{darR}-*gfp*) and pRMJ11 (P_{racD}-*gfp*) were constructed by PCR amplifying the 346-bp intergenic region between VF_1545 and VF_1546 and cloning it between the SphI and SalI sites upstream of *gfp* in pJLS27 (61) using the SphI and SalI sites on primer pairs RJ24 and RJ25 (pRMJ10) or RJ26 and RJ27 (pRMJ11) (Table 1). Complementation plasmids pRMJ8 and pRMJ9 were constructed by amplifying *darR* from ES114 or AN3S2, respectively, with primers RJ57 and RJ58. The resulting PCR products were digested with AvrII and KpnI and ligated into KpnI- and XbaI-digested pVSV105 (62). Complementation plasmid pRMJ15 was constructed by amplifying *racD* from ES114 with primers RJ60 and RJ61. The resulting PCR product was XhoI digested and ligated into SalI-digested pVSV104. Plasmid pRMJ16 was constructed by PCR amplifying the 346-bp region upstream of *racD* using primers RJ26 and RJ27 and cloning this fragment into pCR-Blunt II TOPO (Thermo Fisher, Waltham, MA). Plasmid pRMJ17 was generated from pRMJ16 by replacing the

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TABLE 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics or sequence[a]	Source or reference(s)
Strains		
A. baylyi		
ACN1720	Δ*racD*::*sacB-kanR51720* mutant	This study
ACN1721	Δ*darR*::*sacB-kanR51721* mutant	This study
ACN1725	Δ*racD51725* mutant	This study
ACN1726	Δ*darR51726* mutant	This study
ADP1	Wild type (BD413)	76, 77
E. coli		
BL21-CodonPlus(DE3)-RIPL	B F^- *ompT hsdS*(r_B^- m_B^-) dcm^+ Tet^r *gal*λ(DE3) *endA* Hte [*argU proL* Cam^r] [*argU ileY leuW* Strep/$Spec^r$]	Agilent Technologies, Santa Clara, CA
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA* λ*pir*	59
DH5α	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	78
DH5αλ*pir*	DH5α lysogenized with λ*pir*	63
V. fischeri		
AN3	*murI*::miniTn*5-erm*, D-Glu auxotroph	36
AN3S2	AN3 spontaneous suppressor, *murI*::miniTn*5-erm*, *darR* point mutant (M202I)	This study
ES114	Wild-type isolate from *E. scolopes*	79
RMJ10	Δ*darR* mutant	This study
RMJ12	Δ*racD* mutant	This study
RMJ13	*murI*::miniTn*5-erm* Δ*racD* (VF_1547)	This study
Plasmids[b]		
pBAC1289	*ampR kanR*; *A. baylyi* Δ*racD*::*sacB-kanR51720*; *sacB-kanR* cassette inserted in XhoI site of pBAC1291	This study
pBAC1291	*ampR*; *A. baylyi* Δ*racD51725* in pUC19	This study
pBAC1296	*ampR*; *A. baylyi* Δ*darR1726* in pUC19	This study
pBAC1297	*ampR kanR*; *A. baylyi* Δ*darR*::*sacB-kanR51721*; *sacB-kanR* cassette inserted in XhoI site of pBAC1296	This study
pCR-Blunt II-TOPO	$oriV_{ColE1}$ *kanR*	Thermo Fisher
pEVS94	$oriV_{R6K}$ $oriT_{RP4}$ *ermR*	59
pEVS104	Conjugative helper plasmid; $oriV_{R6K}$ $oriT_{RP4}$ *kanR*	59
pEVS118	$oriV_{R6K}$ $oriT_{RP4}$ *camR*	63
pJLS27	$oriV_{R6K}$ $oriV_{pES213}$ $oriT_{RP4}$ *mCherry kanR* promoterless-*camR-gfp*	61
pRMJ1	*ampR kanR*; source of *sacB-kanR* cassette	66
pRMJ8	*darR* from ES114 cloned into pVSV105	This study
pRMJ9	*darR*(M202I) from AN3S2 cloned into pVSV105	This study
pRMJ10	P_{darR}-*gfp* reporter	This study
pRMJ11	P_{racD}-*gfp* reporter	This study
pRMJ15	*racD* from ES114 cloned into pVSV104	This study
pRMJ16	Region upstream of VF_1546 ES114 cloned into pCR-Blunt II-TOPO	This study
pRMJ17	Synthetic altered DarR target cloned into pCR-Blunt II-TOPO	This study
pRMJ26	Δ*racD* allele in pCR-Blunt II-TOPO	This study
pRMJ27	pRMJ26 ligated to pEVS94	This study
pRMJ30	Δ*darR* allele in pCR-Blunt II-TOPO	This study
pRMJ31	pRMJ30 ligated to pEVS118	This study
pRMJ42	P_{racD}-*gfp* reporter with putative DarR-binding site scrambled	This study
pUC19	$oriV_{ColE1}$ *ampR*; cloning vector	80
pVSV104	$oriV_{R6K}$ $oriV_{pES213}$ $oriT_{RP4}$ *kanR lacZ*α	62
pVSV105	$oriV_{R6K}$ $oriV_{pES213}$ $oriT_{RP4}$ *camR lacZ*α	62
Oligonucleotides[c]		
MTV395	GAGTCAGAGCTCGACATCTTAAAAAAAGCGCATGTG	This study
MTV396	GATCATCTGCAGTGATTCCCTGGATAACGAATTTGAC	This study
MTV397	**CCACGATACTGTCTAGGATCAT**CTCGAGCATTCACCATTTTTGAATTGCCTA	This study
MTV398	**TAGGCAATTCAAAAATGGTGAATG**CTCGAGATGATCCTAGACAGTATCGTGG	This study
MTV399	GAGTCAGAGCTCAAATTGACTGGAAAACATGGTGTTG	This study
MTV400	GATCATCTGCAGCACTTGCTTAGGTACACCAAATT	This study
MTV401	**CTAATAAATATCTTAGATTACAGGTAAAACTCATG**CTCGAGATGAAGGTT CATGCATTTTGTG	This study
MTV402	**CACAAAATGCATGAACCTTCAT**CTCGAGCATGAGTTTTACCTGTAATCTA AGATATTTATTAG	This study
RJ24	GATGCATGCATCATGTCAGTCGGTATGGT	This study
RJ25	CTAGTCGACTTCAACCTCATACAACCATTCT	This study

(Continued on next page)

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TABLE 1 (Continued)

Strain, plasmid, or oligonucleotide	Relevant characteristics or sequence[a]	Source or reference(s)
RJ26	GATGCATGCTTCAACCTCATACAACCATTCTA	This study
RJ27	CTAGTCGACAAAACATCCTATTTAAATTTTAACTAATTGATTTT	This study
RJ35	CGGTAATAATTGAAATCTCAGTAGT	This study
RJ36	AATGCTAGCCATAATATTTACTCCTTTAAATGAAATACGC	This study
RJ37	ATTGCTAGCTAACGATTTCTTCTTACTGAATTCACC	This study
RJ38	CCATATTCATTGGTAGTTCAGTTAT	This study
RJ41	TTTTAATAATACGATCCGGATGAAT	This study
RJ42	AGCGTCGACCATTTCAACCTCATAC	This study
RJ43	CGTGTCGACTAGCTTACGGCATTAT	This study
RJ44	ACTTTTTACCTCTGTTTTCCCACC	This study
RJ57	TAACCTAGGAATGGTTGTATGAGGTTGAAATG	This study
RJ58	AATGGTACCAAGCAAATAATGCCGTAAGCTA	This study
RJ60	AGTCTCGAGGGTGAATTCAGTAAGAAGAAATCG	This study
RJ61	TCACTCGAGGCACTTACTACAACGCGTATTTC	This study

[a]Drug resistance abbreviations used: *camR*, chloramphenicol resistance (*cat*); *ermR*, erythromycin resistance; *kanR*, kanamycin resistance (*aph*); *ampR*, ampicillin resistance; Tet^r, tetracycline resistance; Strep/Spec^r, streptomycin/spectinomycin resistance.
[b]Alleles cloned in this study are from *V. fischeri* strain ES114 or *A. baylyi* strain ADP1. Replication origins (*oriV*) on plasmids are from R6Kγ, ColE1, and/or pES213. Plasmids based on pES213 are stable in *V. fischeri* and do not require antibiotic selection for maintenance (62).
[c]All oligonucleotides are shown in the 5′ to 3′ direction. Underlines indicate restriction enzyme recognition sites. The bold letters correspond to complementary sequences for SOE PCR.

fragment between SphI and SalI restriction sites with a synthesized "BioBrick" DNA fragment (IDT, Coralville, IA) identical to the 346-bp sequence upstream of the VF_1546 translational start site, except that the putative DarR-binding site (5′-ATGCCGATTTGGCAT-3′) from 120 to 135 bp upstream of the gene is replaced with a randomly shuffled sequence (5′-AGTAACCTGTGGTTT-3′), which was arrived at using the Sequence Manipulation Suite (51). Plasmid pRMJ42 was constructed by PCR amplifying the altered intergenic region from pRMJ17 using primers RJ26 and RJ27, digesting this amplicon with SphI and SalI, and cloning into the same sites upstream of *gfp* in pJLS27.

To generate *V. fischeri* mutants, gene deletions were constructed on plasmids, and the corresponding mutants were generated using allelic exchange. A Δ*racD* allele was constructed by amplifying regions upstream or downstream of *racD* with the RJ35 and RJ36 or RJ37 and RJ38 primer pair, respectively. The resulting PCR products were digested with NheI, ligated together, gel purified, and cloned into pCR-Blunt II-TOPO to generate pRMJ26. To facilitate mobilization into *V. fischeri*, pEVS94 (59) and pRMJ26 were each digested with SpeI and ligated together to generate pRMJ27. Plasmid pRMJ27 was conjugated into strains ES114 and AN3, and allelic exchange was confirmed via PCR, resulting in mutants RMJ12 and RMJ13, respectively. A Δ*darR* allele was similarly constructed by amplifying regions upstream or downstream of *darR* with the primer pair RJ41 and RJ42 or RJ43 and RJ44, respectively. The resulting PCR products were digested with SalI, ligated together, gel purified, and cloned into pCR-Blunt II-TOPO to generate pRMJ30. To facilitate allelic exchange in *V. fischeri*, pEVS118 (63) and pRMJ30 were each digested with XbaI and ligated together to generate pRMJ31. Plasmid pRMJ31 was conjugated into strain ES114, and allelic exchange was confirmed via PCR, resulting in Δ*darR* mutant RMJ10.

Mutants of *A. baylyi* ADP1 were constructed using standard methods (64). In some cases, splicing by overlap extension PCR (SOE PCR) (65) was used with the Expand high-fidelity PCR system (Roche) to join DNA fragments. The indicated genomic sequence coordinates for ADP1 correspond to GenBank accession no. CR543861. To delete *racD* (ACIAD0318), PCR was used to amplify wild-type DNA upstream (with primers MTV399 and MTV402) and downstream (with MTV400 and MTV401) of *racD*. These fragments were joined by SOE PCR, and the combined product was digested with SacI and PstI and ligated to similarly digested pUC19. The resulting plasmid, pBAC1291, contains an XhoI site in place of the deleted *racD* sequence. A *sacB-kanR* cassette, excised by digestion with SalI from pRMJ1 (66), was ligated to pBAC1291 digested with XhoI to create pBAC1289. The same process was used to delete *darR* (ACIAD0323), using primer pairs MTV396 and MTV397, and MTV395 and MTV398, to generate pBAC1296. The *sacB-kanR* cassette was introduced into the XhoI site of pBAC1296, which replaced the deleted *darR* sequence, to create pBAC1297.

To introduce the plasmid-borne *A. baylyi* DNA into the chromosome by allelic replacement, naturally competent recipients were transformed with plasmids that were linearized by restriction enzyme digestion, as described previously (67–69). Strains were selected either for resistance to Kan or for the loss of the *sacB* gene in the presence of sucrose (5%). Genotypes were confirmed by PCR and regional DNA sequencing. Linearized pBAC1289 transformed ADP1 to create ACN1720 (Δ*racD*::*sacB-kanR*). Linearized pBAC1291 transformed recipient ACN1720 to obtain ACN1725 (Δ*racD* mutant). Similarly, transformation of ADP1 by linearized pBAC1297 yielded ACN1721 (Δ*darR*::*sacB-kanR* mutant), and transformation of ACN1721 by linearized pBAC1296 yielded ACN1726 (Δ*darR* mutant).

GFP transcriptional reporter assays. Strains harboring green fluorescent protein (GFP)-expressing reporter plasmids were grown in 200 μl of medium in Greiner 96-well flat-bottom black-walled plates (Sigma-Aldrich, St. Louis, MO) with shaking (200 rpm) at 28°C. Optical density at 595 nm (OD_{595}) and red

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and green fluorescence were measured using a Synergy 2 plate reader (Bio-Tek, Winooski, VT). Data presented are from samples where the OD_{595} was between 0.9 and 1.1.

Screen for spontaneous suppressors of D-Glu auxotrophy. Strain AN3 (*murI*::miniTn*5-erm*) was grown to an OD_{595} of 0.5 in LBS supplemented with 400 $\mu g \cdot ml^{-1}$ D-Glu and then dilution plated on LBS without D-Glu. Cultures were dilution plated in parallel on LBS supplemented with D-Glu to determine the total population density. Plates were incubated at 28°C, and colonies were counted at 48 h. Colonies were streak purified, stocked in LBS, and checked for Erm resistance to ensure the presence of the original *murI*::mini-Tn*5-erm* allele.

Peptidoglycan preparation and analysis. To isolate PG, cells were grown to an OD_{595} of ~0.6 in LBS medium, chilled on ice for 10 min, and centrifuged at 4°C and 15,000 × *g* for 10 min. After resuspension in 4 ml cold water, cell suspensions were dripped into 50 ml of boiling 4% SDS with continuous stirring, boiled for 30 min, and allowed to cool to room temperature. Samples were then centrifuged at 130,000 × *g* for 60 min at room temperature, resuspended in 20 ml of 50°C water, and washed twice more in 20 ml of water. The pellet was resuspended in 20 ml of water, heated at 100°C for 2 min to dissolve precipitated SDS, and washed twice more, as described above. Before resuspension, the supernatant was assayed for SDS using methylene blue and chloroform (70). When SDS was undetectable, the pellet was resuspended in 1 ml of 100 mM Tris-HCl (pH 7.5) and subsequently treated with α-amylase (Sigma-Aldrich, St. Louis, MO), DNase I (Sigma-Aldrich), RNase (Sigma-Aldrich), and trypsin (Worthington Biochemical, Lakewood, NJ). SDS was added to 1% and the solution was boiled for 15 min, diluted into 20 ml of warm water, and centrifuged at 130,000 × *g* for 10 min at 20°C. The resulting pellet was washed twice in water, once in 8 M LiCl, and twice more in water. The final pelleted PG was resuspended in 100 μl of water and stored at −80°C. Amino acid (71) and muropeptide (72) analyses were performed by HPLC, as previously described.

Bioinformatic analyses. Putative promoters were predicted using the BPROM tool from Softberry (73) for sequences upstream from *racD*, *darR*, and genes from other bacteria that were near *darR* homologs. The top scoring potential promoters were annotated, and the sequences were trimmed to the −10 region, aligned, and queried for canonical T-N_{11}-A sites using Geneious (57). A visual inspection for dyad symmetry led to the selection of a new query (5′-ATG-N_9-CAT-3′). This sequence appeared upstream of all six putative promoters examined, including the putative DarR-binding site in *V. fischeri* described above. Further examination of these sequences led to the proposed 5′-ATGC-N_7-GCAT-3′ binding site, and this site was queried against genome databases (sometimes allowing a 1-bp mismatch) using Pattern Locator (74). The genomic context of *darR* homologs was found by searching the RAST database for genes similar to *darR* using the SEED Viewer version 2.0 with a window of 16 kbp (75). The DarR homologs depicted beneath *V. fischeri* DarR (VF_1545) in Fig. 6 from top to bottom correspond to the following annotated open reading frames: BDGL002587 (*Acinetobacter calcoaceticus*), ACIAD0323 (*A. baylyi* ADP1), STM4511 (*Salmonella enterica*), Dd586_2631 (*Dickeya dadantii*), ETAE_0919 (*Edwardsiella tarda*), F382_06960 (*Mannheimia haemolytica*), PBPRA2355 (*Photobacterium profundum*), B4327 (*E. coli* MG1655 K-12), Z5926 (*E. coli* O157:H7), VV2_1132 (*Vibrio vulnificus*), STM4511 (*Salmonella enterica*), VF_1510 (*V. fischeri*), PBPRB1351 (*P. profundum*), Pfl01_5624 (*Pseudomonas fluorescens*), Psyr_5050 (*Pseudomonas syringae*), ACAID1745 (*A. baylyi* ADP1), CV_4113 (*Chromobacterium violaceum*), SPO2668 (*Ruegeria pomeroyi*), Meso_4540 (*Mesorhizobium* sp. strain BNC1), Bpro_3905 (*Polaromonas* sp. strain JS666), Psyr_2713 (*Pseudomonas syringae*), BP0764 (*Bordetella pertussis*), RSc0479 (*Ralstonia solanacearum*), and BMA2440 (*Burkholderia mallei*).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00773-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.2 MB.

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