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RESEARCH ARTICLE

Transposon and Deletion Mutagenesis of Genes Involved in Perchlorate Reduction in *Azospira suillum* PS

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ABSTRACT **Although much work on the biochemistry of the key enzymes of bacterial perchlorate reduction, chlorite dismutase, and perchlorate reductase has been published, understanding of the molecular mechanisms of this metabolism has been somewhat hampered by the lack of a clear model system amenable to genetic manipulation. Using transposon mutagenesis and clean deletions, genes important for perchlorate reduction in *Azospira suillum* PS have been identified both inside and outside the previously described perchlorate reduction genomic island (PRI). Transposon mutagenesis identified 18 insertions in 11 genes that completely abrogate growth via reduction of perchlorate but have no phenotype during denitrification. Of the mutants deficient in perchlorate reduction, 14 had insertions that were mapped to eight different genes within the PRI, highlighting its importance in this metabolism. To further explore the role of these genes, we also developed systems for constructing unmarked deletions and for complementing these deletions. Using these tools, every core gene in the PRI was systematically deleted; 8 of the 17 genes conserved in the PRI are essential for perchlorate respiration, including 3 genes that comprise a unique histidine kinase system. Interestingly, the other 9 genes in the PRI are not essential for perchlorate reduction and may thus have unknown functions during this metabolism. We present a model detailing our current understanding of perchlorate reduction that incorporates new concepts about this metabolism.**

IMPORTANCE **Although perchlorate is generated naturally in the environment, groundwater contamination is largely a result of industrial activity. Bacteria capable of respiring perchlorate and remediating contaminated water have been isolated, but relatively little is known about the biochemistry and genetics of this process. Here we used two complementary approaches to identify genes involved in perchlorate reduction. Most of these genes are located on a genomic island, which is potentially capable of moving between organisms. Some of the genes identified are known to be directly involved in the metabolism of perchlorate, but other new genes likely regulate the metabolism in response to environmental signals. This work has uncovered new questions about the regulation, energetics, and evolution of perchlorate reduction but also presents the tools to address them.**

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Over billions of years, bacteria have evolved the ability to reduce dozens of different electron acceptors as part of their energy metabolism, from widely distributed molecules, such as oxygen, nitrate, and sulfate, to more esoteric compounds, like perchlorate. Naturally occurring perchlorate is thought to have an atmospheric origin but is deposited only in very small quantities (1); it accumulates mainly in hyperarid environments, such as the Atacama Desert and the Antarctic Dry Valleys (2). Anthropogenic perchlorate, however, is a by-product of various industries and can lead to localized contamination of groundwater due to its chemical stability and aqueous solubility (3). Despite the sporadic accumulation of perchlorate in the extant terrestrial environment, dissimilatory perchlorate-reducing bacteria (DPRB) are ubiquitous and can be isolated from many different environments (4).

Perchlorate reduction by microorganisms has been observed for decades but was initially thought to be a product of the nitrate reduction machinery common to many eubacteria and archaea (5). Further study of pure-culture DPRB isolates revealed two enzymes specific to this metabolism: perchlorate reductase (PcrAB) and chlorite dismutase (Cld). PcrAB is a heterodimeric molybdoenzyme that belongs to the widespread and functionally diverse dimethyl sulfoxide (DMSO) reductase family (6). PcrAB reduces perchlorate to chlorite, which is then dismutated into chloride and oxygen by the heme-containing Cld (7, 8). The chlorite dismutase gene was initially identified in the chlorate reducer *Ideonella dechloratans* and subsequently in the DPRB *Dechloromonas agitata* CKB (9, 10). In the regions flanking the *cld* gene in two *Dechloromonas* species, the genes for the molybdopterin-containing perchlorate reductase were found (6). More recently, the entire genomes of several DPRB have been sequenced, and all share a perchlorate reduction genomic island (PRI) that includes *pcrAB* and *cld* in addition to several conserved genes with unknown function (11). Similarly, a comparative study of chlorate reducers found that chlorate reduction genes are associated with composite transposons which contain conserved genes with unknown function (12). These comparative genomic studies pro-

vided further evidence that (per)chlorate reduction genes are transferred horizontally, and several new genes were identified in the PRI that could be involved in regulation of the metabolism (11). Previous efforts to understand the regulation of perchlorate reduction by environmental conditions have used the enzymatic activity of whole cells or extracts, but little is known about the genetic or transcriptional basis for these observations (13, 14).

While continued isolation of novel DPRB provides a diverse collection of bacteria capable of this metabolism (15–17), understanding the basic tenets of perchlorate reduction is hindered by the lack of a robust model system amenable to genetic manipulation. To fill this role, we have selected the betaproteobacterium *Azospira suillum* PS (formerly known as *Dechlorosoma suillum*) (18). PS is a facultative anaerobe that is capable of respiring perchlorate and nitrate under anoxic conditions. It grows rapidly and robustly on rich media, and its genome has been fully sequenced and is publicly available (11, 19). Additionally, its perchlorate-reducing physiology has been studied in the context of environmental regulation (13), chemotaxis (20), and redox cycling of electron shuttles (21, 22).

In this study, we set out to broaden our knowledge about the molecular mechanisms and genetic factors involved in perchlorate reduction. We performed a random transposon mutagenesis of PS and identified 18 mutants unable to grow on perchlorate, most of which contained transposon insertions in the PRI. To further dissect this genomic island, we developed a method for creating markerless deletions and constructed in-frame knockout mutants for the 17 core genes in the PRI. We found that eight were essential for perchlorate reduction, while the remaining nine genes were either partially defective during growth on perchlorate or had no obvious phenotype. As expected, *pcrABCD* and *cld* were essential for perchlorate reduction, but so were genes for a response regulator (*pcrR*), histidine kinase sensor (*pcrS*), and a PAS domain-containing protein (*pcrP*), which form a putative regulatory system. Complementation of all eight genes using plasmid-carried copies of the deleted gene resulted in partial or complete restoration of growth via perchlorate reduction. This study expands the list of genes essential for perchlorate reduction but also supplies the tools for further analysis of the genetics and regulation of this metabolism.

RESULTS

Transposon mutagenesis of PS and screening for mutants. Transposon mutagenesis and screening identify the genetic factors involved in a given phenotype in an unbiased manner. We mutagenized PS using a plasmid-based MiniHimar transposon, which was delivered using the conjugation-competent *Escherichia coli* strain WM3064 (23, 24). Mutants were isolated as single, kanamycin-resistant colonies aerobically on a medium designed to maximize the breadth of genes disrupted during mutagenesis. This medium contained acetate, lactate, and pyruvate as carbon sources and was thus designated ALP media (see Materials and Methods).

To screen the mutants for defects associated with perchlorate reduction, we took advantage of the fact that PS grows rapidly in the presence of both nitrate and perchlorate when transferred from aerobic conditions (Fig. 1). Additionally, when both nitrate and perchlorate are present under anaerobic conditions, nitrate is used in preference to perchlorate by this organism, resulting in a diauxic growth curve (13). The mutant library was screened for

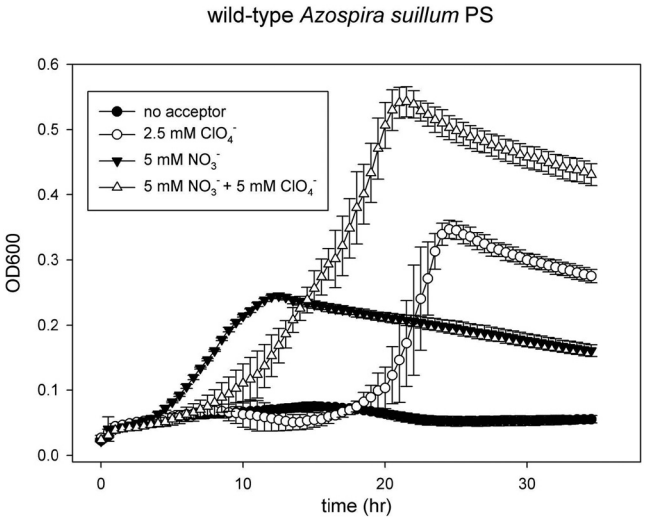

FIG 1 PS growth on nitrate and perchlorate. Wild-type PS was grown on medium containing nitrate, perchlorate, and a mixture of nitrate and perchlorate.

growth under these conditions over a 30-h period. Individual mutants that exhibited defects in either growth rate or final optical density were restreaked and selected for further study. To identify mutants with perchlorate-specific defects, each mutant was further characterized for the ability to grow on nitrate, perchlorate, nitrate/perchlorate, and oxygen. Many mutants that were selected for additional characterization displayed wild-type growth characteristics, suggesting that the initial screen had a high incidence of false positives. Some mutants were pleiotropic, suffering defects of various intensities in both growth rate and final optical density under multiple conditions. In order to focus on mutants directly involved in perchlorate reduction, we defined a subset of the mutants as "perchlorate null" mutants. These mutants did not grow at all on perchlorate but exhibited normal growth on nitrate alone, as well as normal aerobic growth on both solid and liquid ALP media. The full phenotypic characterizations of the transposon mutants of interest are detailed in Table 1. In total, 18 perchlorate null mutants and 18 pleiotropic mutants were isolated (Table 1).

Transposon mutagenesis highlights importance of genes in the PRI. The genomic locations of transposon insertions were identified using an arbitrary PCR-based method (Table 1). Many pleiotropic mutations in genes predicted to be important for anaerobic respiration, including genes for molybdopterin biosynthesis (*moeA* and *mogA*), molybdenum uptake (*modC*), nitrate reduction/denitrification (*napA*, *norD*, and Dsui_1177), anaerobic heme biosynthesis (*hemN*), and ubiquinone biosynthesis (*ubiX*), were identified. Additionally, three insertions in the *rnf* operon resulted in wild-type growth on nitrate and oxygen but very slow growth on perchlorate. Similarly, an insertion in a gene from the *relA*/*spoT* family (Dsui_2816) was associated with a very low growth rate on perchlorate.

While the pleiotropic mutants had insertions in genes located throughout the chromosome, most (14 out of 18) of the perchlorate null mutant insertions were localized to 8 genes in the PRI (11). Insertions were found in previously identified structural genes (*pcrA*, *pcrB*, *pcrD*, and *cld*) but also in several genes of un-

TABLE 1 Transposon mutants mapped and characterized in this study[a]

Transposon mutant ID	Locus tag of insertion-containing gene	Gene name and gene product	Phenotype	Growth on nitrate	Growth on perchlorate	Growth on nitrate and perchlorate	Aerobic growth
C4H3	Dsui_0128	D-Alanyl–D-alanine carboxypeptidase	Perchlorate null	++	0	+	++
A4F1	Dsui_0145	*cld*; chlorite dismutase	Perchlorate null	++	0	0	++
B1H6	Dsui_0145	*cld*; chlorite dismutase	Perchlorate null	++	0	0	++
C5B11	Dsui_0145	*cld*; chlorite dismutase	Perchlorate null	++	0	0	++
B9B7	Dsui_0146	*pcrD*; perchlorate reductase cytoplasmic chaperone	Perchlorate null	++	0	+	++
B9G5	Dsui_0148	*pcrB*; perchlorate reductase beta subunit	Perchlorate null	++	0	+	++
C5A2	Dsui_0148	*pcrB*; perchlorate reductase beta subunit	Perchlorate null	++	0	+	++
A3G1	Dsui_0149	*pcrA*; perchlorate reductase alpha subunit	Perchlorate null	++	0	+	++
A5D9	Dsui_0149	*pcrA*; perchlorate reductase alpha subunit	Perchlorate null	++	0	+	++
A7B10	Dsui_0149	*pcrA*; perchlorate reductase alpha subunit	Perchlorate null	++	0	+	++
A4A8	Dsui_0150	*pcrR*; PRI response regulator	Perchlorate null	++	0	+	++
A4A9	Dsui_0151	*pcrS*; PRI histidine kinase	Perchlorate null	++	0	+	++
B7G4	Dsui_0151	*pcrS*; PRI histidine kinase	Perchlorate null	++	0	+	++
C1D2	Dsui_0152	*pcrP*; PRI PAS domain protein	Perchlorate null	++	0	+	++
A9A5	Dsui_0153	PRI cupin domain protein	Perchlorate null	++	0	+	++
A5A6	Dsui_0704	*rpoN*; sigma 54-type sigma factor RpoN	Perchlorate null	++	0	+	++
B4D1	Dsui_0704	*rpoN*; sigma 54-type sigma factor RpoN	Perchlorate null	++	0	+	++
A7E8	Dsui_1441	Phenylacetate-CoA ligase[b]	Perchlorate null	++	0	+	++
B4E5	Dsui_0261	*cysP*; periplasmic binding protein of sulfate/thio-sulfate ABC transporter	Pleiotropic null	+	+	+	++
A2D6	Dsui_0508	*napA*; nitrate reductase alpha subunit	Pleiotropic null	+	0	0	++
A8E1	Downstream of Dsui_0508	*napA*; nitrate reductase alpha subunit	Pleiotropic	+	+	+	++
B6F5	Dsui_0564	*modC*; ATPase subunit of molybdate ABC transporter	Pleiotropic	++	+	+	++
A8H2	Dsui_0675	*dsbA*; thiol:disulfide interchange protein	Pleiotropic	+	+	+	++

(Continued on following page)

TABLE 1 (Continued)

Transposon mutant ID	Locus tag of insertion-containing gene	Gene name and gene product	Phenotype	Growth on nitrate	Growth on perchlorate	Growth on nitrate and perchlorate	Aerobic growth
A9G11	Dsui_0993	*ubiX*; 3-octaprenyl-4-hydroxybenzoate carboxylase	Pleiotropic	+	+	+	++
B7F10	Dsui_1054	*trpB*; tryptophan synthase	Pleiotropic	++	+	+	++
A7B1	Dsui_1177	Heme *b*/*c*-containing EbdC-like electron transport protein	Pleiotropic	0	+	+	++
B2H11	Dsui_1236	*mogA*; molybdopterin adenylyltransferase	Pleiotropic	+	0	+	++
B3E6	Dsui_1533	*dsbB*; disulfide bond formation protein	Pleiotropic	+	++	+	++
A3C3	Dsui_1578	*aceE*; pyruvate dehydrogenase	Pleiotropic	+	+	+	+
C1A4	Dsui_1647	*hemN*; oxygen-independent coproporphyrinogen III oxidase	Pleiotropic	+	+	+	++
B9B5	Dsui_2152	*moeA*; molybdopterin molybdotransferase	Pleiotropic	+	+	+	++
C4E9	Dsui_2816	(p)ppGpp synthetase, RelA/SpoT family	Pleiotropic	++	+	+	++
C5B9	Upstream of Dsui_2979	*rnfA*; Rnf electron transport complex alpha subunit	Pleiotropic	++	+	+	++
B9H11	Dsui_2979	*rnfA*; Rnf electron transport complex alpha subunit	Pleiotropic	+	+	+	++
B1F10	Dsui_2982	*rnfD*; Rnf electron transport complex delta subunit	Pleiotropic	++	+	+	++
B2D12	Dsui_3119	*norD*; nitric oxide reductase activation protein	Pleiotropic	+	++	+	++
A5B2	Dsui_2880	*bioA*; adenosylmethionine-8-amino-7-oxononanoate transaminase	No phenotype (control)	++	++	++	++

[a] Transposon mutants were scored "++" if growth was identical to that of the wild type, "+" if growth rate or final optical density was less than that of the wild type, or "0" if no growth was observed.
[b] CoA, coenzyme A.

known function. Three genes in the PRI identified in this screen encode a putative histidine kinase signal transduction system (HKS). We have named these genes *pcrR*, *pcrS*, and *pcrP* to reflect their linkage with *pcrA* and the fact that homologs of some or all of these genes can be identified in the PRI of other perchlorate reducers, some of which are distantly related to PS (11). The gene names *pcrR*, *pcrS*, and *pcrP* are intended to be suggestive of the putative roles of each gene product in the HKS: the response *r*egulator, the kinase *s*ensor, and the *P*AS domain-containing protein. PcrP and PcrS are predicted to be integral membrane proteins with large regions in both the cytoplasm and periplasm. The cytoplasmic region of PcrS contains domains seen in many bacterial histidine kinases: a HAMP linker domain (PF00672), a phosphoacceptor domain (PF00512), and an ATPase domain (PF02518) (25). PcrP contains two PAS domains from different subfamilies in its cytoplasmic region (PF08448 and PF13188) (26). In contrast to the cytoplasmic regions, which are composed of well-characterized and common domains, the periplasmic regions of PcrP and PcrS are not homologous to any domains in the COG or Pfam databases. These regions are fairly large, consisting of approximately 250 and 270 amino acids in PcrS and PcrP, respectively. BLAST-P searches of the PcrP domain against finished genomes identified several similar sequences, while a search using the PcrS domain identified only the PcrS homolog from another DPRB, *Dechloromonas aromatica* RCB. PcrR contains three domains commonly found in response regulators: the receiver domain (PF00072), a σ_{54} interaction domain (PF00158), and a helix-turn-helix DNA-binding domain (PF02954) (27). Another insertion in a perchlorate null mutant mapped to the cupin domain protein gene (Dsui_0153) in the PRI. Outside the PRI, three genes also contained insertions that led to the perchlorate null phenotype. Two separate mutants with insertions in Dsui_0704,

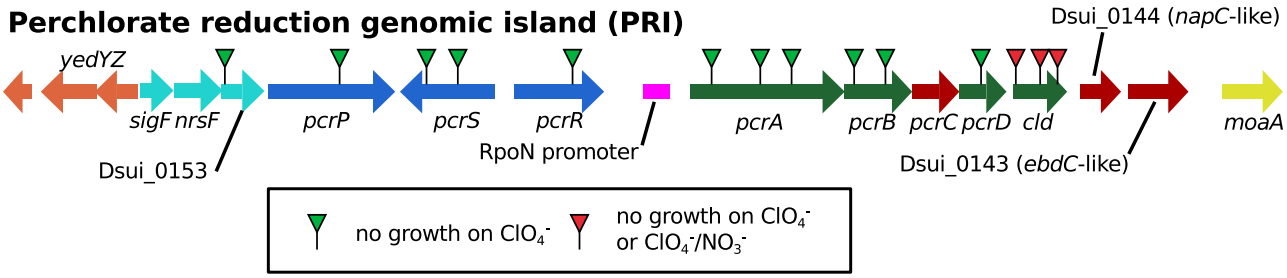

FIG 2 Locations of transposon insertions in the PRI. The 17 genes in the PRI are displayed with the locations of transposon insertions that were identified in the screen. The green pins indicate mutants that did not grow on perchlorate, while the red pins indicate mutants that did not grow on perchlorate or on the diauxic medium. Genes are colored by functional group: green genes encode chlorite dismutase and perchlorate reductase components, royal-blue genes are the PRI HKS, red genes are parts of the putative electron transport chain, orange genes are components of a putative oxidoreductase system, and light-blue genes are part of a putative sigma factor/antisigma system.

which encodes the alternative σ_{54}-type sigma factor RpoN, were isolated. The other two genes (Dsui_0128 and Dsui_1441) are of unknown function.

Two diauxic phenotypes associated with "perchlorate null" mutants. Perchlorate null mutants were defined as having no growth on medium containing perchlorate, while being indistinguishable from wild-type PS on medium with nitrate. When the 18 perchlorate null mutants were grown on diauxic medium containing nitrate and perchlorate, two different phenotypes were observed. Most (15 out of 18) of the perchlorate null mutants had similar growth curves on diauxic medium and nitrate-only medium. The remaining three perchlorate null mutants had significantly impaired growth on diauxic medium relative to that on medium containing only nitrate. Interestingly, all three of these mutants had transposon insertions in *cld*, the gene encoding chlorite dismutase (Fig. 2). The other 15 perchlorate null mutants had insertions in 10 different genes, including *pcrA* (Fig. 2). To highlight the different diauxic growth phenotypes, *pcrA*::Himar and *cld*::Himar transposon mutants were compared to a biotin biosynthetic mutant (*bioA*::Himar) which had wild-type growth characteristics on kanamycin-amended ALP medium, presumably due to the presence of biotin in the vitamin supplement (Fig. 3A to C). Neither the *pcrA*::Himar nor the *cld*::Himar mutants grew at all on perchlorate (Fig. 3B), but the *cld*::Himar mutant grew to a much lower optical density than the *pcrA*::Himar mutant on nitrate and perchlorate (Fig. 3C).

Developing a genetic system in PS and systematic deletions of genes in the PRI. To confirm that genes identified in the transposon screen were essential for perchlorate reduction, we developed a system for making markerless chromosomal deletions in PS. Several different suicide vectors did not undergo chromosomal integration when delivered via conjugation, but a suicide vector derived from pNPTS138 (Dickon Alley, unpublished data) was able to integrate into the chromosome. However, conjugation proved to be an unpredictable method of vector delivery, since conjugations would frequently fail and no chromosomal insertions could be recovered. Reliable chromosomal insertions of vectors derived from pNPTS138 were achieved using electroporation, although efficiency of integration was low (5 to 10 insertions per μg plasmid DNA transformed). This is consistent with the previous observation that *Azospira* spp. are resistant to transformation (28).

We used pNPTS138 to create deletions of the 17 genes in the conserved core of the PRI. Because many genes in the PRI are in operons (e.g., *pcrABCD*), we designed the deletion constructs to create in-frame deletions of each gene that did not disturb the ribosomal binding site or promoters of nearby genes. Once each deletion was made, we characterized the strain's ability to grow on nitrate, perchlorate, and nitrate plus perchlorate. Of the eight genes in the PRI that were defined as being perchlorate null mutants during the transposon screen, seven (*pcrABD*, *cld*, and *pcrPSR*) recapitulated the perchlorate null phenotype. Additionally, the deletion of *pcrC* resulted in a perchlorate null phenotype, although no insertions in *pcrC* were isolated during the transposon screen. When Dsui_0153 was deleted, the resulting strain exhibited no differences from the wild type under all conditions tested, suggesting that the perchlorate null phenotype of Dsui_0153::Himar was the result of a polar effect, possibly on the adjacent *pcrP* gene.

In addition to Dsui_0153, deletion of six other genes in the PRI also had no apparent phenotype. Five of these genes (Dsui_0154 to Dsui_0158) are situated at one end of the PRI and comprise two oppositely oriented operons. The five genes contain a predicted sigma factor/anti-sigma factor pair, genes homologous to *yedYZ* from *E. coli* (29), and a gene encoding a hypothetical protein. The sixth nonessential gene (Dsui_0141) is located at the opposite end of the PRI and is a homolog of *moaA*, a gene involved in molybdopterin biosynthesis (30). Two additional genes in the PRI, Dsui_0143 and Dsui_0144, were not essential for perchlorate reduction, but both mutants suffered from slight yet significant growth rate defects on perchlorate. Dsui_0144 is predicted to encode a tetraheme *c*-type cytochrome homologous to proteins such as NapC, NirT, and CymA, all of which function as quinol dehydrogenases in electron transport chains involved in the reduction of various electron acceptors (31). Dsui_0143 is predicted to be a gene encoding a protein homologous to EbdC, the gamma subunit of ethylbenzene dehydrogenase of *Aromatoleum aromaticum*, which contains a heme *b* and forms a heterotrimer with the alpha and beta subunits (32). However, the Dsui_0143 protein may contain two additional heme *c* molecules, since it contains two putative heme *c* binding motifs (one CXXCH motif and an atypical CWXXCH). These two motifs are conserved in the homologous NirB protein, which is encoded in a cluster of genes involved in nitrite reduction in *Pseudomonas stutzeri* (33). The growth phenotypes for all 17 single deletions are summarized in Table 2.

Complementation of perchlorate reduction mutants. To show that the eight genes from the PRI were independently essential for perchlorate reduction and the observed phenotypes were

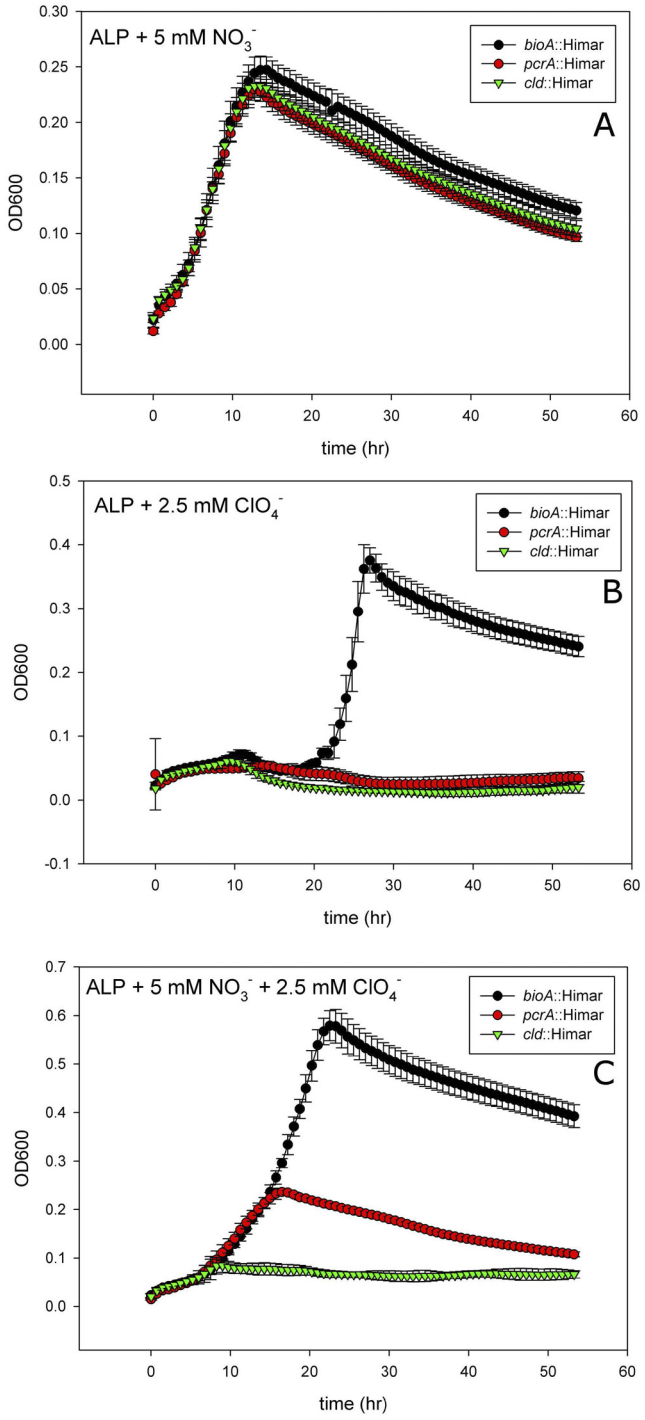

FIG 3 Growth of *pcrA*::Himar and *cld*::Himar strains. The growth of strains with transposon insertion mutations in *cld*, *pcrA*, and *bioA* (control) shows distinct phenotypes on nitrate (A) or perchlorate (B) or under diauxic conditions (C).

not the result of polar effects, we developed a system for complementation analysis. The broad-host-range plasmid pBBR1MCS2 was tested for stable replication in PS and was used as the backbone for all complementation vectors. The complementation vectors were constructed in two groups. The first group contained the genes *pcrPSR*, which are assumed to be transcribed independently based on their alternating orientation (Fig. 2). When constructing these vectors, we included the ~150-bp sequence upstream of the start codon in order to retain the native promoter and regulation. The second group contains five essential genes (*pcrABCD* and *cld*) located in the same operon. To construct these vectors, we first made a plasmid with the *pcrA* promoter (pRAM62) and then incorporated each of the five genes independently into pRAM62 with their endogenous ribosomal binding site. Each of the eight perchlorate null deletion mutants received both the complementation vector and the empty pBBR1MCS2 vector. Under anaerobic conditions with both nitrate and perchlorate, all eight complemented strains were able to grow on perchlorate, while the strains with the empty vector recapitulated the defective growth curves seen in the deletion mutants (see Fig. S1 to S3 in the supplemental material). The complemented strains had variable lag times when switching from nitrate to perchlorate and different growth rates on perchlorate. The reasons for this are unknown but could be due to differences in expression between the complemented mutants and wild-type PS.

Phenotypic characterization of Δ*cld* Δ*pcrA* double mutant. To further explore the discrepancy between the diauxic phenotypes of Δ*cld* and Δ*pcrA*, we constructed a Δ*cld* Δ*pcrA* double mutant by electroporating the *cld* deletion vector into the previously constructed Δ*pcrA* strain. As previously described, the Δ*cld* strain exhibited normal growth on nitrate alone but was impaired for growth on nitrate when perchlorate was also present (Fig. 4A and B). We hypothesized that this was due to baseline expression of *pcrA* and accumulation of chlorite in the absence of Cld activity. As expected, the Δ*cld* Δ*pcrA* strain did not grow on perchlorate but was able to grow normally on nitrate (Fig. 4A and C). However, in the presence of nitrate and perchlorate, the Δ*cld* Δ*pcrA* mutant recovered the ability to grow on nitrate as did the Δ*pcrA* single deletion mutant (Fig. 4B).

DISCUSSION

Previous work identified the *cld* gene by sequencing a fragment of a purified enzyme with chlorite dismutase activity (34). This sequence was used to amplify and sequence *cld* from *Dechloromonas agitata* CKB and subsequently used to identify neighboring perchlorate reductases (6, 10). *pcrA* was directly mutagenized and shown to be essential in *Dechloromonas aromatica* RCB; however, this was done with an antibiotic cassette insertion and may have resulted in polar effects on nearby genes (6). A forward genetics approach confirmed that *cld* and *pcrA* are essential for perchlorate reduction in *Azospira suillum* PS and also identified the importance of *pcrB*, *pcrD*, a new HKS, and *rpoN*. Markerless in-frame deletion was used to confirm the insertions in the PRI and also led to the identification of *pcrC* as an essential gene for perchlorate reduction. These complementary genetic approaches advance our understanding of the genetic factors involved in perchlorate reduction and establish *Azospira suillum* PS as a model perchlorate reducer.

The majority of the genes identified as essential for perchlorate reduction are located in the perchlorate reduction genomic island (PRI), which contains 17 genes in PS that are conserved in *Dechloromonas aromatica* RCB but not in other closely related organisms incapable of perchlorate reduction (11). Our transposon screen identified *cld*, *pcrA*, *pcrB*, and *pcrD* as essential for perchlorate reduction, which we confirmed with deletion mutagenesis. Al-

TABLE 2 Annotations of the 17 core PRI genes and phenotypic information based on deletion mutants[a]

Locus tag	Gene name	Gene product	Pfam domain(s)	Growth on perchlorate	Growth on nitrate and perchlorate
Dsui_0141	*moaA*	Molybdenum cofactor biosynthesis protein	PF04055, PF06463, PF13353	++	++
Dsui_0143		Heme *b*/*c*-containing EbdC-like electron transport protein	PF09459	+	++
Dsui_0144		Quinol dehydrogenase tetraheme cytochrome *c* (NapC-like)	PF03264	+	++
Dsui_0145	*cld*	Chlorite dismutase	PF06778	0	0
Dsui_0146	*pcrD*	Perchlorate reductase cytoplasmic chaperone	PF02613	0	+
Dsui_0147	*pcrC*	Tetraheme cytochrome *c*	PF13435	0	+
Dsui_0148	*pcrB*	Perchlorate reductase beta subunit	PF13247	0	+
Dsui_0149	*pcrA*	Perchlorate reductase alpha subunit	PF00384, PF01568	0	+
Dsui_0150	*pcrR*	PRI response regulator	PF00072, PF00158, PF02954 0	0	+
Dsui_0151	*pcrS*	PRI sensor histidine kinase	PF00512, PF00672, PF02518 0	0	+
Dsui_0152	*pcrP*	PRI PAS domain protein	PF08448, PF13188	0	+
Dsui_0153		Cupin domain protein	PF07883	++	++
Dsui_0154	*nrsF*	Anti-sigma factor	PF06532	++	++
Dsui_0155	*sigF*	ECF sigma factor	PF04542, PF08281	++	++
Dsui_0156	*yedZ*	Cytochrome *b* membrane electron transport protein	PF00033	++	++
Dsui_0157	*yedY*	Unknown molybdopterin oxidoreductase	PF00174	++	++
Dsui_0158		Hypothetical protein		++	++

[a] For growth on perchlorate, "++" denotes wild-type growth on perchlorate, "+" denotes growth at a lower rate than that of the wild type, and "0" denotes no growth. For growth on nitrate and perchlorate, "++" denotes wild-type growth, "+" denotes growth only during the initial denitrification phase, and "0" denotes very little growth. All deletion mutants grew identically to the wild type on nitrate alone.

though it was not identified in the transposon screen, *pcrC* was also found to be essential for perchlorate reduction. *pcrB* encodes the beta subunit of the perchlorate reductase, and the initial biochemical characterization of perchlorate reductase from *Azospira* sp. GR-1 identified a stable enzyme complex in an $\alpha_3\beta_3$ conformation (8). PcrB is likely required for the reductase complex stability and for delivering reducing equivalents from the inner membrane electron transport chain to the active site of PcrA. PcrC is a multiheme cytochrome *c* that was previously thought to be part of the perchlorate reduction pathway in all DPRB (6), but a recently isolated and described organism, *Arcobacter* sp. CAB, reduces perchlorate and does not have a gene homologous to *pcrC* in its PRI or elsewhere in its genome (35). *pcrD* encodes a putative chaperone that is homologous to both TorD and NarJ of *E. coli* and has been predicted to be the chaperone for PcrA (6, 36, 37). Many periplasmic molybdoenzymes of the DMSO reductase family have dedicated chaperones which bind to the signal peptide and prevent transport of the apoprotein via the Tat (*t*win-*a*rginine *t*ranslocation pathway) until addition of the appropriate cofactor (37, 38). The perchlorate null phenotype of the Δ*pcrD* strain suggests that PcrD may similarly be required for the attachment of molybdopterin to PcrA and the proper translocation of the holoenzyme.

pcrP, *pcrR*, and *pcrS* were identified both in the transposon screen and by deletion mutagenesis as being essential for perchlorate reduction. Together, their gene products comprise a histidine kinase signal transduction system that is conserved in other DPRB (11). The sensory inputs and physiological outputs of this system are currently unknown, but the DNA-binding domain of PcrR suggests that it likely binds to promoters to modulate transcription. PcrR also contains a σ_{54} interaction domain, which can recruit σ_{54}-type sigma factors to promoters where the regulator is bound and hydrolyze ATP to initiate transcription (39). PS contains exactly one σ_{54}-type sigma factor, encoded by the *rpoN* gene (Dsui_0704), which was one of only three genes outside the PRI that resulted in a perchlorate null phenotype when mutated with a transposon insertion. Additionally, there is a conserved promoter region upstream of *pcrA* that contains an RpoN-binding motif (11). Based on these observations, we hypothesize that at least one of the outputs of this system is to upregulate transcription of the *pcrABCD* operon in an RpoN-dependent manner. The presence of two separate sensor proteins that are both essential (PcrP and PcrS) hints at a complex regulatory mechanism.

The periplasmic domains of PcrP and PcrS are unique, and the signals that modulate this system are unknown. Perchlorate, nitrate, or intermediates of both reductive pathways could be sensed directly, as is the case for the *E. coli* NarX sensor kinase, which autophosphorylates in response to binding nitrate at the dimerization interface (40). An emerging paradigm for histidine kinase "two-component" signal transduction is the presence of a third auxiliary protein which can adjust the autophosphorylation of the histidine kinase and/or phosphotransfer to the response regulator (41). PcrP could be such an auxiliary protein, and it could sense signals that originate either in the periplasm or in the

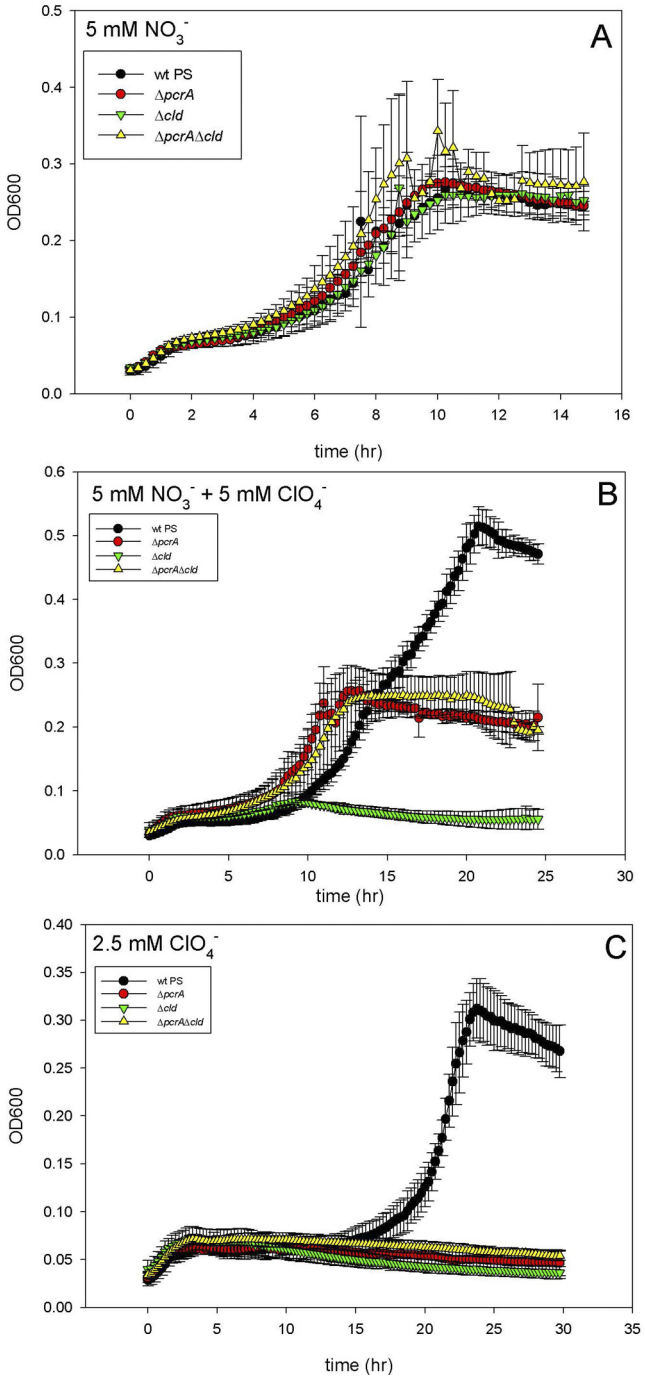

FIG 4 Growth characteristics of wild-type PS and the Δ*cld*, Δ*pcrA*, and Δ*cld* Δ*pcrA* strains. Growth on nitrate (A), under diauxic conditions (B), or on perchlorate (C) shows that a Δ*cld* Δ*pcrA* double mutant grows better on mixed electron acceptors than the Δ*cld* and Δ*pcrA* single mutants.

cytoplasm, perhaps via its two PAS domains. Interestingly, the *pcrPSR* genes do not appear in the genome of *Dechloromonas agitata* CKB, a DPRB that does not respire nitrate, unlike PS, suggesting that the PRI HKS could play a role in nitrate-dependent repression of perchlorate reduction (11, 13, 42). We have presented a schematic for the basic functions of the PRI HKS, highlighting the domain structure of PcrP and PcrS in the inner membrane, as well as the putative function of PcrR in initiating transcription in an RpoN-dependent manner (Fig. 5). Although we currently know little about how the system functions, molecular analysis of the signals and domains involved will be crucial in understanding the evolution of the PRI.

Nine genes in the PRI were not required for perchlorate reduction (Dsui_0141, Dsui_0143-Dsui_0144, and Dsui_0153 to Dsui_0158). All of these genes are conserved in at least one other DPRB, and thus it was unexpected that so many (9 out of 17) had no obvious role in perchlorate reduction (11). However, a similar observation was made in a genetic study of the magnetosome genomic island, where many of the conserved genes were not essential for magnetosome formation (43). Possible reasons proposed for this phenomenon were genetic redundancy, as well as the inherent limitations of laboratory-imposed growth conditions (43). It is reasonable to expect that both of these caveats are relevant for genetic analysis of the PRI.

The perchlorate reductase receives electrons from the electron transport chain via an unknown pathway. PS has a predicted quinol dehydrogenase-type cytochrome *c* (Dsui_0144) encoded in its PRI that was previously proposed to be a conduit from the quinone pool to the reductase (6), but there was only a slight impairment of growth on perchlorate when the gene was deleted in PS. A similar phenotype was seen with the deletion of the predicted cytochrome *b/c* gene Dsui_0143. Because none of these genes are absolutely essential for perchlorate reduction, we propose that the electron transport chain to PcrA is redundant. This sort of multiplicity is seen on a larger scale in organisms such as *Geobacter* spp., which utilize dozens of different cytochrome *c* proteins to transport electrons across the cell envelope to multiple electron acceptors (44). The perchlorate electron transport chain may not be quite as complex, but additional genetic and biochemical evidence is needed to resolve this pathway in PS. The limitations of our experimental growth conditions may also occlude the phenotypes of certain genes in the PRI. For example, Dsui_0154 and Dsui_0155 are predicted to encode an extracytoplasmic function sigma factor/anti-sigma factor system homologous to SigF and NrsF of *Caulobacter crescentus*, which has been shown to play a role in activating a response to reactive oxygen species and heavy metal stress (45, 46). The phenotype of these knockouts in PS may be apparent only under the appropriate oxidative stress conditions, which are not met by our standard acceptor-limited conditions in rich medium.

One possible source of oxidative stress associated with perchlorate reduction could be an intermediate of the metabolism. Both the transposon screen and deletion mutagenesis revealed that the *cld* mutant had a different phenotype from the rest of the perchlorate null mutants when grown on nitrate and perchlorate together (Fig. 3). Interestingly, deletion of *pcrA* in the Δ*cld* background relieved the growth inhibition (Fig. 4). In the context of perchlorate reduction, chlorite dismutase liberates molecular oxygen from chlorite and sustains the microaerobic respiration that is thought to accompany perchlorate reduction (5). However, Cld can also function to detoxify chlorite, which has been proposed as a role for Cld in organisms that cannot reduce perchlorate or chlorate (47). Chlorite is a powerful oxidant that can result in cell death even at parts-per-million concentrations (48). Previous experiments with PS indicate that perchlorate is not reduced with nitrate in large quantities, but if perchlorate reductase is expressed at all under nitrate-reducing conditions in the absence of Cld, the

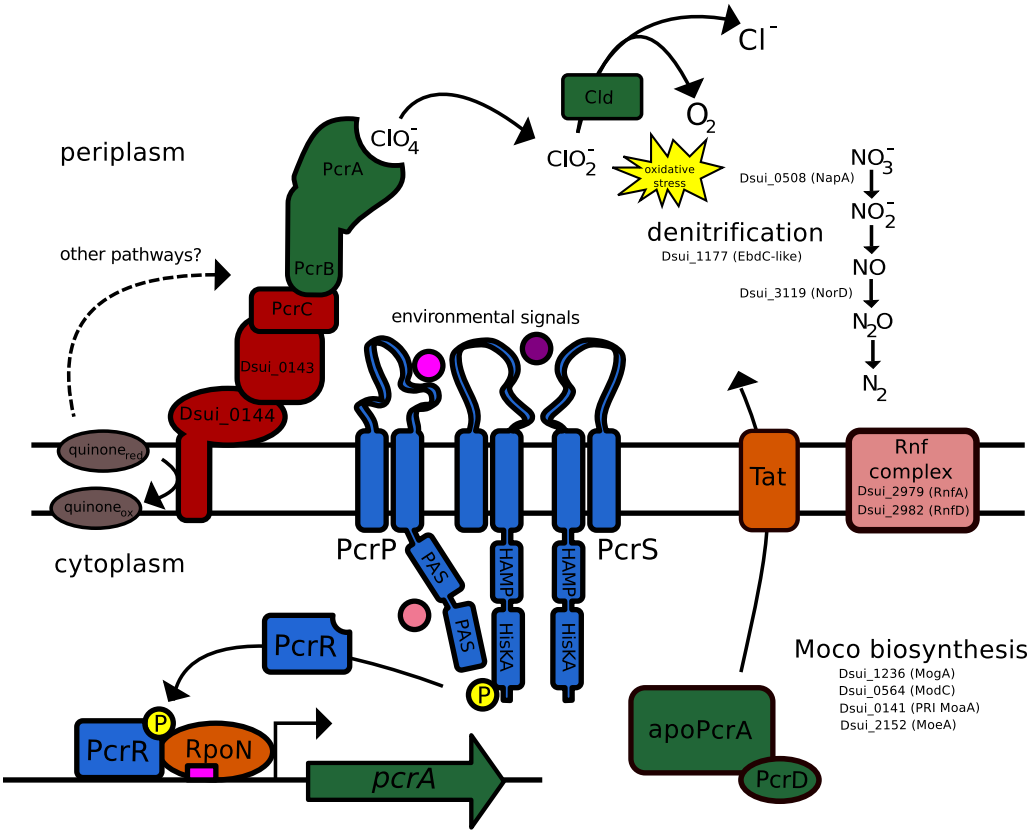

FIG 5 A model of perchlorate reduction in *Azospira suillum* PS. Gene products from the PRI are depicted in a model that reflects our current understanding of perchlorate reduction and its regulation in *Azospira suillum* PS. Several genes identified in the transposon screen as important for perchlorate reduction are also shown (molybdopterin cofactor biosynthesis, denitrification, and the Rnf complex). Gene products from the PRI are colored to be consistent with the functional group coloring in Fig. 2.

micromolar amounts of chlorite that accumulate may result in the inhibition of cell growth. A study performed with cell extracts of the closely related DPRB *Azospira* sp. KJ grown on nitrate showed some perchlorate reductase activity, suggesting that there could be baseline expression of PcrA even under nitrate-reducing conditions (14).

As previously proposed, perchlorate reduction is dependent on genes in the horizontally transferred PRI but also on genes elsewhere in the genome (11). Our transposon screen identified several operons and pathways that are involved in anaerobic respiration. Insertions in *hemN* (Dsui_1647) and *ubiX* (Dsui_0993) resulted in pleiotropic mutants that were defective in growth on both nitrate and perchlorate. HemN is an oxygen-independent coproporphyrinogen III oxidase, and UbiX is a decarboxylase involved in the biosynthesis of quinones (49, 50). Presumably, these mutants are deficient in anaerobic heme and quinone biosynthesis, which leads to a general growth defect under anaerobic conditions. Perchlorate reductase requires a molybdopterin-bound molybdenum to function, and several genes involved in molybdopterin biosynthesis were identified in the transposon screen (Table 1). These mutants were also wholly or partially defective during denitrification, which is likely due to the nitrate reductase utilizing the same cofactor. Additionally, the transposon screen allowed the identification of several genes putatively involved in denitrification, which has not been studied directly in PS. These genes include *napA*, *norD*, and Dsui_1177. The gene product of Dsui_1177 is a heme-containing protein which has been shown to be upregulated under denitrifying conditions (21). Interestingly, the *napA* and Dsui_1177 mutants also demonstrate some defects in perchlorate reduction. The reason for this is currently unknown.

Many other genes were identified in the transposon screen as being partially defective in perchlorate reduction and not denitrification, but their specific functions are difficult to predict. These include the *rnf* operon, which was hit by transposons several times in the mutagenesis and screen. The *rnf* operon encodes a membrane-bound redox-active complex which couples the proton (or sodium) gradient of the inner membrane to the cellular redox pools of NAD^+/NADH and ferredoxin (51). The activity of the Rnf complex is reversible and has been shown to be important in an array of different bacterial metabolisms, although its role in perchlorate reduction is currently unknown. Other transposon insertions are in genes that are not directly linked to cellular energetics or are in genes that encode hypothetical proteins. Exploring the roles of these genes will be essential to gaining a holistic understanding of perchlorate reduction in the context of global cellular metabolism.

In this article, we set out to address a basic question about the molecular biology of perchlorate reduction: which genes are essential for this metabolism? Transposon mutagenesis and a subsequent screen on medium containing nitrate and perchlorate identified many mutants with insertions in the PRI, accentuating

its importance. To further characterize this region, we developed a system for making markerless in-frame deletions and systematically knocked out each gene in the PRI. The results of these complementary genetic approaches have increased our understanding of the factors involved in perchlorate reduction (Fig. 5) but have raised many new questions. The histidine kinase system in the PRI is essential for perchlorate reduction, although we know little about how it is activated or which genes it regulates. Specifically, utilization of nitrate over perchlorate is still not understood. The components that deliver electrons to PcrA are also unknown, as is the role played by the nonessential genes in the PRI. Finally, the evolution of the PRI is still obscure; how does the island integrate with the host's metabolism after it is acquired? Genetic analysis using the deletion and complementation tools developed here will be critical to answering these questions about the evolution and function of the PRI in *Azospira suillum* PS and thus other DPRB.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Azospira suillum* PS (ATCC BAA-33/DSMZ 13638) was revived from a lab freezer stock and used as the wild-type strain for all genetic manipulations (see Table S2 in the supplemental material). Various *E. coli* strains were also used for cloning and conjugation purposes (see Table S2). Plasmids constructed or used in this study are described in Table S3. Prior to all growth curves and genetic manipulations, all strains were streaked out from master freezer stocks to get single colonies.

Culture conditions and growth media. *E. coli* strains were grown in LB medium. Kanamycin (Kan) (50 μg/ml) was used for selection, and diaminopimelic acid (DAP) (0.3 mM) was used as a supplement to cultivate the auxotrophic strain WM3064. For routine culturing, as well as growth assays, wild-type and mutant strains of PS were grown in ALP medium. One liter of ALP medium is composed of 0.49 g monobasic sodium phosphate dihydrate, 0.97 g dibasic anhydrous sodium phosphate, 0.1 g potassium chloride, 0.25 g ammonium chloride, 0.82 g sodium acetate, 2.0 g yeast extract, 7.6 g of a 60% (wt/wt) sodium lactate solution, 1.10 g sodium pyruvate, and 10 ml of both vitamin mix and mineral mix as previously described (42). To make solid ALP medium for plates, 15 g/liter agar was added. Kanamycin was used for selection of PS mutants at a concentration of 50 μg/ml. For anaerobic growth of PS, ALP medium was supplemented with either 5 mM sodium nitrate, 2.5 mM sodium perchlorate, or 5 mM of both sodium nitrate and sodium perchlorate. No growth was observed when no electron acceptor was added, confirming that PS is unable to grow via fermentation of the carbon sources present in ALP medium. All strains of *E. coli* and PS were cultivated at 37°C. Anaerobic growth curves of PS for the transposon screen and characterization of strains was performed using a SpectraMax 340PC384 plate reader in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI).

Transposon mutagenesis via conjugation. The *mariner* transposon suicide vector pMiniHimar RB1 was used to mutagenize PS (23). WM3064 containing pMiniHimar RB1 (A. Arkin lab, University of California [UC], Berkeley) was grown as overnight culture in 5 ml LB plus Kan plus DAP. This culture was centrifuged and washed twice in ALP medium to remove excess kanamycin. In parallel, a 5-ml culture of PS was grown from a single colony overnight (optical density at 600 nm [OD_{600}] of ~1.5) and centrifuged. The WM3064 and PS cell pellets were resuspended in 50 μl ALP medium and spotted onto an ALP plate supplemented with DAP. The plate was dried by an open flame for 15 min on the benchtop to ensure that the agar surface was dry and the mating reaction did not spread. The plate was then incubated at 37°C for 6 h, after which the entire spot was scraped off the plate and resuspended in 1 ml ALP medium. The volume was divided, plated on 6 ALP-Kan plates, and incubated at 37°C. After 36 to 48 h, ~300 to 500 whitish-pink opaque colonies formed on the 6 plates in total. The colonies were then transferred into 96-well plates containing 300 μl of ALP-Kan medium supplemented with 7.5% glycerol as a cryoprotectant. The plates were then grown overnight aerobically on a platform shaker at 37°C prior to screening and long-term storage at −80°C.

Screening the transposon mutant library. The transposon library was screened prior to long-term storage. From each glycerol freezer stock plate, 10 μl was transferred into 300 μl of ALP-Kan medium supplemented with 5 mM nitrate and perchlorate on the benchtop. Plates were then transferred into the anaerobic chamber, where they were allowed to equilibrate with the anaerobic atmosphere for 1 h. An initial optical density reading was taken, and the plates were placed in a GasPak box (BD) with a palladium catalyst for maintenance of anaerobic conditions. The box was removed from the glove bag and incubated at 37°C. Every 6 to 8 h for 30 h, the box was moved back into the glove bag and the optical density of the plates was measured. The growth curve data were plotted, and mutants that had a defect in either growth rate or maximum optical density were identified. The "mutants of interest" were then streak purified to isolate single colonies on ALP-Kan medium. The single colonies were grown on ALP medium to make long-term freezer stocks.

Characterization of transposon mutants defective in perchlorate reduction. Mutants of interest identified in the initial screen were subjected to further phenotypic characterization. Each mutant was streaked out on ALP-Kan plates from freezer stocks and then transferred into an overnight culture of ALP-Kan liquid medium. The next morning, the optical densities of all strains were determined, and each culture was diluted back to an OD_{600} of 1.0 to 1.2. A 50-μl aliquot was used to inoculate 1.45 ml of ALP-Kan medium supplemented with either nitrate (5 mM), perchlorate (2.5 mM), or nitrate and perchlorate (both 5 mM). The concentration of perchlorate under the perchlorate-only condition was lowered to avoid unpredictably long lag periods seen with high perchlorate concentrations (unpublished data). Each medium condition was distributed to 4 wells in a 96-well plate such that 8 genotypes could be assayed simultaneously using the SpectraMax plate reader in the anaerobic chamber. The characterization growth curves were allowed to run for 48 h.

Mapping the locations of transposon mutants. Transposon mutants of interest were mapped using a variation of arbitrarily primed PCR methods previously described (52, 53). Briefly, transposon mutants of interest were streaked onto ALP-Kan plates, and single colonies were used as templates for colony PCR. The first round of PCR used a primer that annealed to the end of the *mariner* transposon (HIMAR_EXT) (see Table S1 in the supplemental material) and one of three arbitrary primers (PS_ARB4, PS_ARB5, and PS_ARB6) which contain a conserved 20-mer at the 5′ end, followed by 10 random nucleotides and one of three most common pentamers in the *A. suillum* PS genome. This first round of PCR used the GoTaq Green master mix (Promega) and the following thermocycling parameters: 10 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 38°C, and 2 min at 72°C; and 10 min at 72°C. The reaction mixture was treated with ExoSAP-IT (Affymetrix) to remove the primers, and the product was used as the template for a second round of PCR using a transposon primer closer to the genomic insertion site (HIMAR_INT) and a primer consisting of the conserved 20-mer at the 5′ end of the arbitrary primers (PS_ARB2). The second round of PCR used the following thermocycling parameters: 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 55°C, and 2 min at 72°C; and 10 min at 72°c. The second-round PCR product was purified using the QIAquick PCR purification kit (Qiagen) and used as the template for a sequencing reaction with the HIMAR_INT primer (UC Berkeley DNA Sequencing Facility). Sequencing reads were manually curated and used as queries for searching the *A. suillum* genome with BLAST on the NCBI server (54). Sequences were downloaded, and the exact insertion site of the transposon in each mutant was determined.

Constructing suicide vectors and complementation vectors. Genomic DNA from *A. suillum* was isolated using Trizol according to the manufacturer's directions (Invitrogen), and all PCRs were performed using Phusion DNA polymerase (Fisher Scientific). Specific primer pairs were designed using the NCBI Primer-BLAST server to amplify products

of 800 to 900 bp flanking the gene to be deleted (55). The internal primers closest to the gene were designed to end between codons within the gene so that the resulting deletion would be in-frame and have minimal polar effects on nearby genes. For some genes, the internal primers had a 21-bp "linker" at the 5′ end that was used to assemble the flanking regions into a 1.6- to 1.8-kb insert using PCR. For the remaining genes, the internal primers contained restriction sites that allowed assembly of the suicide vector with a three-way ligation. Both methods used resulted in an in-frame deletion allele of the gene of interest, thereby minimizing polar effects on nearby genes. Sequences and brief descriptions of all primers are listed in Table S1 in the supplemental material.

We used the vector pNPTS138 (Dickon Alley via Kathleen Ryan, UC, Berkeley) as the suicide vector backbone for all of our deletion constructs after testing several plasmids with no success (pAK31, pK19mobsacB, and pSMV3). Deletion vectors were constructed by amplifying two regions flanking the gene of interest and either performing an assembly PCR to fuse the regions together or digesting the PCR product directly. In both cases, the cut vector and digested PCR product(s) was digested with the appropriate restriction enzymes, analyzed by electrophoresis on an agarose gel, and extracted using the Qiaex II gel extraction kit (Qiagen). The digested products were then ligated into the vector using T4 ligase (NEB). The broad-host-range plasmid pBBR1MCS2 was used as the backbone for the complementation vectors (56). For three genes (*pcrP*, *pcrS*, and *pcrR*), pBBR1MCS2 and the amplified gene with its promoter were digested, purified, and ligated as described above. For the *pcrABCD* and *cld* complementation vectors, the *pcrA* promoter was first cloned into pBBR1MCS2 to make pRAM62. The amplified genes were then cloned downstream of this promoter as described above to generate these complementation vectors. All ligation reactions were transformed into chemically competent *E. coli* TOP10 cells and plated on selective medium. Individual colonies were grown overnight in liquid broth, and plasmid DNA was purified. Plasmids were checked for the presence of the correct insert by digestion with appropriate restriction enzymes, followed by sequencing of the insert (UC, Berkeley DNA Sequencing Facility).

Electroporation of suicide vectors into PS and screening of deletions. Electrocompetent *A. suillum* PS cells were prepared using a method previously described for *Zymomonas mobilis* (57). One-hundred-microliter aliquots of electrocompetent cells were thawed on ice and mixed with 1 μg of plasmid DNA and 0.8 μl of TypeOne restriction inhibitor (Epicentre Biotechnologies). The mixture was transferred into a cuvette on ice and then electroporated at 1,750 V, 400 Ω, and 25 μF. Immediately after electroporation, 300 μl of ALP medium was added to the cuvette, and the entire aliquot was transferred to a sterile 1.7-ml tube for 6 h of recovery in a 37°C shaking incubator. After the recovery, the aliquot was plated on 3 ALP-Kan plates and incubated at 37°C for 36 h. Colonies that formed within 36 h were picked into 500 μl of ALP-Kan liquid medium and grown for 12 h to confirm integration of the suicide vector into the chromosome. An aliquot of 50 μl was then transferred into 5 ml of ALP medium and grown overnight. Dilutions of the overnight culture were plated on ALP plates with 6% sucrose (ALP-Suc) to select against the *sacB* allele present on the pNPTS138 backbone and incubated at 37°C for 36 h. Colonies that appeared were patched onto ALP-Kan and ALP-Suc and incubated at 37°C overnight. Colonies that were sensitive to kanamycin and resistant to sucrose were used as a template for colony PCR using primers flanking the gene to be deleted. The primers used for each gene were the external primers used previously to amplify the flanking regions of the deletion construct. The PCR products were analyzed using gel electrophoresis on a 1% agarose gel, and colonies that yielded a PCR product with the correctly sized deleted allele size were restreaked on an ALP plate. Single colonies from the restreaked plate were checked once again with colony PCR for the deleted allele and then picked into an overnight ALP medium culture to make into freezer stocks.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00769-13/-/DCSupplemental.

Figure S1, EPS file, 0.8 MB.
Figure S2, EPS file, 0.6 MB.
Figure S3, EPS file, 0.8 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.

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