Control of Proteobacterial Central Carbon Metabolism by the HexR Transcriptional Regulator

A CASE STUDY IN SHEWANELLA ONEIDENSISS

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Bacteria exploit multiple mechanisms for controlling central carbon metabolism (CCM). Thus, a bioinformatic analysis together with some experimental data implicated the HexR transcriptional factor as a global CCM regulator in some lineages of Gammaproteobacteria operating as a functional replacement of the Cra regulator characteristic of Enterobacteria. In this study, we combined a large scale comparative genomic reconstruction of HexR-controlled regulons in 87 species of Proteobacteria with the detailed experimental analysis of the HexR regulatory network in the Shewanella oneidensis model system. Although nearly all of the HexR-controlled genes are associated with CCM, remarkable variations were revealed in the scale (from 1 to 2 target operons in Enterobacteriales up to 20 operons in Aeromonadales) and gene content of HexR regulons between 11 compared lineages. A predicted 17-bp palindrome with a consensus tTGTAATwwwATTACa was confirmed as a HexR-binding motif for 15 target operons. A dual mode of HexR action on various target promoters, repression of genes involved in catabolic pathways and activation of gluconeogenic genes, was for the first time predicted by the bioinformatic analysis and experimentally verified by changed gene expression pattern in S. oneidensis hexR mutant. Phenotypic profiling revealed the inability of this mutant to grow on lactate or pyruvate as a single carbon source. A comparative metabolic flux analysis of wild-type and mutant strains of S. oneidensis using [13C]lactate labeling and GC-MS analysis confirmed the hypothesized HexR role as a master regulator of gluconeogenic flux from pyruvate via the transcriptional activation of phosphoenolpyruvate synthase (PpsA).

Fine-tuning of the carbohydrate catabolic pathway expression is key to successful adaptation of microorganisms that occupy niches with variable carbon availability. Escherichia coli and related Enterobacteria utilize two global transcription factors (TFs), Crp and Cra (FruR), to control central and peripheral carbohydrate metabolism (1). The cAMP receptor protein, Crp, mediates catabolic repression of target genes in the presence of high levels of glucose, a preferable carbon source for Enterobacteria. In the presence of cAMP, Crp positively regulates its target genes by binding the cognate DNA sites. cAMP is generated by adenylate cyclase that is activated by components of the glucose-specific phosphotransferase system in the absence of glucose (2). The LacI protein family carbobolate repressor/activator, Cra, was initially characterized as the fructose repressor, FruR, because it negatively regulates transcription of the fructose utilization operon (3). Later, Cra was characterized as a pleiotropic transcriptional regulator that

Footnotes:

†The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S4 and Figs. S1–S4.

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§The abbreviations used are: TF, transcription factor; EMP pathway, Embden Meyerhof-Parnas pathway; ED pathway, Entner-Doudoroff pathway; KDPG, 2-keto-3-deoxy-6-phosphogluconate; FPA, fluorescence polarization assay.

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represses multiple genes from the central glycolytic pathways, namely the EMP and ED pathways, and activates genes involved in gluconeogenesis and oxidative phosphorylation (4). DNA binding activity of Cra is triggered by two central intermediates of the EMP pathway, fructose 1-phosphate and fructose 1,6-bisphosphate. Comparative genomic analysis of the Cra (FruR) regulon revealed substantial variability in regulon gene content among Enterobacterales and that it acted as a local regulator of the fructose utilization operon in other γ-proteobacteria such as *Vibrio* and *Pseudomonas* species (5).

A different regulatory strategy is utilized to control central carbohydrate metabolism in the genus *Pseudomonas*, which favors the utilization of organic acids and amino acids over various other carbon sources (6). In contrast to Enterobacteria, the *Pseudomonas* EM glycolytic pathway is nonfunctional due to the absence of 6-phosphofructokinase (7). Degradation of glucose proceeds ultimately via the ED pathway, where 6-phosphogluconate and KDPG are key intermediates. Expression of all genes encoding the ED pathway in *Pseudomonas putida*, including glucokinase (*glk*), glucose-6-phosphate dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*), 6-phosphogluconate dehydrogenase (*edd*), KDPG aldolase (*eda*), and glyceraldehyde-3-phosphate dehydrogenase (*gap-1*), is negatively regulated by HexR (8, 9). Two monomers of HexR bind to imperfect palindromic sites with consensus sequence of TTGTN7–8ACAA in the promoter regions of the *zwf*, *edd*, and *gap-1* genes. Binding of the ED pathway intermediate KDPG to HexR releases the repressor from its target sites (8–11).

Previously, we have applied the integrated genomic and experimental approaches to predict and validate novel metabolic pathways and transcriptional regulons involved in carbohydrate utilization in the *Shewanella* genus of γ-proteobacteria (12, 13). The obtained genomic encyclopedia of sugar utilization included 17 distinct peripheral pathways with committed TFs (*e.g.*, the N-acetylglucosamine utilization pathway controlled by NagR regulon) and a core set of central carbohydrate metabolism genes, of which 12 genes were predicted to be controlled by the HexR regulator (Fig. 1B). In this study, we expand

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**FIGURE 1.** Metabolic context of the reconstructed HexR regulons in Proteobacteria (A) and *S. oneidensis* MR-1 (B). Numbers in blue circles show the number of genomes where the target gene is preceded by a candidate HexR-binding site.
Proteobacterial HexR Catabolite Repressor/Activator

this analysis toward comparative genomic reconstruction of the HexR regulon in all sequenced Proteobacteria. We report the identification of DNA-binding motifs for HexR regulators and provide a detailed description of corresponding regulons in the genomes of 62 γ-proteobacteria and 25 β-proteobacteria. The comparative analysis of reconstructed regulons revealed considerable variability in the regulon content between the analyzed Proteobacteria. By correlating the binding site position within promoter regions with expression patterns of downstream genes, it was possible to predict the activation mode for HexR regulation of several target genes in *Shewanella* spp. A combination of *in vivo* and *in vitro* experimental techniques was used to validate the predicted HexR-dependent regulatory network in *Shewanella oneidensis* MR-1.

**EXPERIMENTAL PROCEDURES**

Bioinformatics Techniques and Resources—Genomic sequences were obtained from GenBank™ (14). Identification of orthologs and gene neighborhood analysis were performed in Microbes OnLine (15). Functional annotations of genes involved in central carbohydrate metabolism and related pathways were derived from the SEED comparative genomic database (16). Phylogenetic trees were built using the maximum likelihood method in the PHYLPACK Package and visualized with Dendroscope (17). Sequence alignments were made with MUSCLE (18). Distant homologs were identified using pBLAST (19).

We used a well established comparative genomics method of regulon reconstruction (reviewed in Ref. 5) implemented in the RegPredict webserver (regpredict.lbl.gov) (20). We started from the genomic identification of a reference set of genomes that encodes HexR orthologs (according to the phylogenetic analysis of HexR proteins on supplemental Fig. S1). To find the conserved DNA-binding motif for HexR in each group of phylogenetically related genomes, we used initial training sets of known HexR targets from *Pseudomonas* spp., and then we updated each set by the most likely HexR-regulated genes confirmed by the comparative genomics tests as well as the functional and genome context considerations.

In each of 13 studied groups of Proteobacteria, an iterative motif detection procedure implemented in the RegPredict web tool was used to identify common regulatory DNA motifs in a set of upstream gene fragments and to construct the motif recognition profiles as described previously (21). For each clade of HexR proteins in the phylogenetic tree, we used a separate training gene set. The initial recognition profile was used to scan the genomes in this clade and to predict novel genes in the regulon. Scores of candidate sites were calculated as the sum of positional nucleotide weights. The score threshold was defined as the lowest score observed in the training set. For each conserved DNA-binding motif, the respective group profile was rebuilt to improve search accuracy. Sequence logos for the derived group-specific DNA-binding motifs were drawn using WebLogo package (22).

DNA Binding Assays—Interaction of purified recombinant-tagged HexR protein with its cognate DNA-binding sites in *S. oneidensis* MR-1 was assessed by two techniques, electrophoretic mobility shift assay and fluorescence polarization assay, using two sets of synthetic double-stranded oligonucleotides 3′-labeled with either biotin or 6-carboxyfluorescein, respectively. The dsDNA fragments containing the predicted HexR-binding sites were obtained by annealing custom-synthesized complementary oligonucleotides at a 1:1 ratio of labeled to unlabeled complementary oligonucleotides (supplemental Table S1).

For EMSAs, the 49-bp biotin-labeled dsDNA fragments (0.1 nm) were incubated with 500 nm purified HexR in a total volume of 20 μl as described previously (27). The binding buffer contained 20 mm Tris-HCl (pH 8.0), 150 mm KCl, 5 mm MgCl₂, 1 mm DTT, 1 mm EDTA, 0.05% Nonidet P-40, and 2.5% glycerol. Poly(dI-dC) was added as a nonspecific competitor DNA at ~10⁴-fold molar excess over labeled target DNA to reduce nonspecific binding. After 25 min of incubation at 37 °C, the reaction mixtures were separated by electrophoresis on a 5%
native polyacrylamide gel in 0.5× Tris borate/EDTA (100 min, 90 V, room temperature). The DNA was transferred by electrophoresis (30 min at 380 mA) onto a Hybond-N+ membrane and fixed by UV cross-linking. Biotin-labeled DNA was detected with the LightShift chemiluminescent EMSA kit. The effect of phosphorylated intermediates of the ED pathway on HexR-DNA binding was tested by addition of 2-keto-3-deoxy-6-phosphogluconate, glucose 6-phosphate, 6-phosphogluconate, and phosphoenolpyruvate to the incubation mixture (2 mM).

For FPA assays, the 27-bp fluorescence-labeled dsDNA fragments (1 nm) were incubated with the increasing concentrations of purified HexR (10–1000 nM) in 100 μl of reaction mixture in 96-well black plates (VWR, Radnor, PA). Binding buffer contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.3 mg/ml BSA, and 1 μg of poly(dl-dC). Fluorescence measurements were taken on Beckman DTX 880 multimode plate reader with excitation and emission filters at 495 and 520 nm, respectively. Background fluorescence from buffer was subtracted, and the fluorescence intensities measured in the parallel and perpendicular directions were taken as a percentage of the fluorescence polarization value defined as follows: \[ FP = \left( \frac{I_1 - G \cdot I_2}{I_1 + G \cdot I_2} \right), \]
where \(I_1\) and \(I_2\) are the fluorescence intensities measured in the parallel and perpendicular directions, respectively, in the orientation of the excitation polarization, and \(G\) is a correction factor.

Phenotypic Analysis and Metabolite Measurements—Chromosomal in-frame deletion mutants of hexR (SO2490) or ppsA (SO2644) genes were constructed using previously published methods (13). S. oneidensis MR-1 wild type (WT) and ΔhexR and ΔppsA mutant strains were precultured on LB medium to late exponential growth phase, and washed with M1 minimal medium without any carbon sources. Duplicate 50-ml cultures were inoculated to the same absorbance (OD600) genes were constructed using previously published methods (13).

**RESULTS**

Genomic Reconstruction of HexR Regulons in Proteobacteria

Phylogenetic Distribution of hexR Orthologs—Orthologs to the P. putida hexR were identified by BLAST searches against a nonredundant set of sequenced bacterial genomes and used to analyze the phylogenetic relationships between these proteins.
The experimentally studied diverged Alteromonadales and Oceanospirillales genomes. Two highly to lineages of fulfill the essential glucokinase function, and the role of its with the somal arrangement is retained in the transcriptional regulator and its target genes) often occur close on related genes (supplemental Table S2). Among the teobacteria, hexR date HexR-binding sites are shown by regulated genes in representative bacterial genomes. (supplemental Fig. S1). A single copy of Proteobacterial HexR Catabolite Repressor/Activator of hexR bacteria. The containing operons is different in some genomes (Fig. 2). A mono- is co-localized on the chromosome with glucokinase, hexR and pyruvate kinase, edd-eda were found in the Alteromonadales lineage (3). Similar four-gene operons encoding the complete ED pathway served in other Enterobacteriales with the exception of P. putida hexR/H9253 -proteobacteria, although the content of hexR is missing in all of the Pasteurellales, Xanthomonadales, and Moraxellaceae lineages and in some Alteromonadales and Oceanospirillales genomes. Two highly diverged hexR paralogs were detected in Pseudomonadales. The experimentally studied P. putida paralog PP1021 is similar to hexR genes from other γ-proteobacteria, whereas the second paralog (termed hexR1) is more related to hexR from β-proteobacteria. The Burkholderia genomes also possess a second copy of hexR gene, which is translationally fused to the glucokinase gene, glk. Because of the absence of other glk homologs in Burkholderia, the chimeric hexR2-glk gene is the only candidate to fulfill the essential glucokinase function, and the role of its HexR2 domain is to be elucidated.

Chromosomal Co-localization of hexR Genes—Functionally related genes (e.g. genes involved in the same pathway or a transcriptional regulator and its target genes) often occur close on the bacterial chromosome (32). Indeed, the previously characterized in P. putida hexR regulator is divergently transcribed with the zwf-pgl-eda operon (9). Similar divergent chromosomal arrangement is retained in the Pseudomonas and other lineages of γ-proteobacteria, although the content of zwf-containing operons is different in some genomes (Fig. 2). A monocystronic zwf gene previously detected in E. coli (33) is conserved in other Enterobacteriales with the exception of Edwardsiella tarda, which encodes a zwf-pgl-edd-eda operon. Similar four-gene operons encoding the complete ED pathway were found in the Alteromonadales lineage (e.g. in all Sheewanella spp.). We also noted that in many γ-proteobacteria, hexR is co-localized on the chromosome with glucokinase, glk, and pyruvate kinase, pykA. Among β-proteobacteria, hexR is divergently transcribed with edd-eda in Burkholderia or it belongs to either zwf-hexR operon in Comamonadaceae or the zwf-pgl-glk-hexR-pgi operon in Neisseria spp. This analysis further confirmed functional coupling between hexR and the central carbohydrate metabolism genes and allowed us to define a training set of upstream gene sequences for identification of HexR binding motifs.

Identification of HexR-binding Sites and Regulons—To reconstruct the HexR regulons in Proteobacteria, we applied the integrative comparative genomics approach (as implemented in RegPredict webserver (20)) that combines identification of TFs and candidate transcription factor-binding sites with cross-genomic comparison of regulons and with the genomic and functional context analysis of candidate target genes. First, we collected training sets of prospective target genes for each out of 11 defined taxonomic groups possessing HexR orthologs. These training sets were initially defined by the analysis of chromosomal co-localization of hexR genes and a previous knowledge of the HexR regulon in P. putida and then were further expanded by identifying orthologous target genes in the analyzed genomes. For each taxonomic group, we extracted DNA upstream regions of putative operons in this training set and applied a transcription factor-binding site motif recognition program to derive conserved HexR-binding motifs. After construction of a positional weight matrix for each identified motif, we searched for additional HexR-binding sites in the analyzed genomes, and we finally performed a consistency check or cross-species comparison of the predicted regulons (reviewed in Ref. 5). For those groups of genomes where the HexR regulon was expanded by multiple novel candidate target genes, the above procedure was repeated to include these targets into the transcription factor-binding site motif model and to revise the final gene content of the regulon.

A highly conserved palindromic signal with consensus TGTARnnnnnYTACA (where R and Y denote purines and pyrimidines, respectively) was identified as a candidate HexR-binding motif in nine groups of analyzed genomes (supplemental Fig. S1). Two different motifs were detected for two groups of HexR paralogs in the Pseudomonas lineage. Three previously characterized HexR-binding sites in P. putida coincide with the binding motif for the HexR group inferred in this study (consensus sequence aTGTGT4 – 8 nucleotides-ACaAcAt). The candidate binding motif for the second group of regulators in Pseudomonas (HexR1) is similar to HexR-binding motifs in other Proteobacteria. Significant difference between the predicted binding motifs of HexR paralogs in Pseudomonas suggests that the respective regulons should not cross-talk with each other. A different conserved motif, which does not have a palindromic symmetry but which has some resemblance to a right part of the classical HexR-binding motif, was predicted for HexR regulons in Hahella and Marinobacter spp.

The content of the reconstructed HexR regulons in 11 taxonomic groups of γ- and β-proteobacteria (totally 87 species) is summarized in supplemental Table S3. Detailed information about the predicted DNA-binding sites and downstream regulated genes is provided in the RegPrecise database (regprecise.lbl.gov) (23). The reconstructed HexR regulons control the central carbohydrate metabolism in all analyzed proteobacteria (Fig. 1A). However, as we describe it below, the specific content of HexR regulons is highly variable between different lineages.

Conserved Members of HexR Regulons—Based on overall appearance and taxonomic distribution of HexR-regulated genes, we classified them into several groups (Table 1). The first group of genes includes the most conserved regulon members that are regulated by HexR in at least 30 species from at least six different lineages. This group includes zwf, pgi, edd, eda, gapA, pykA, glk, and pgi genes that encode key glycolytic enzymes from the ED and EMP pathways. In addition, the hexR gene was...
predicted to be autoregulated in most genomes. The second
group includes genes that belong to the HexR regulons in at
least 10 species from at least two lineages. Genes in the second
group are involved in glycolysis (\textit{gpmM} and \textit{tpiA}), gluconeo-
genesis (\textit{ppsA}, \textit{gapB}, and \textit{pckA}), the pentose phosphate pathway
(\textit{tal}), pyruvate metabolism (\textit{aceEF} and \textit{ppc}), fermentation
(\textit{adhE}, \textit{pfBA}, and \textit{gcrA}), glyoxylate metabolism (\textit{aceBA}), amino
acid biosynthesis (\textit{gltBD}), and NADPH re-oxidation (\textit{pntAB}).
The third group includes genes that were found within the
HexR regulons in two or more lineages but appeared in less
than 10 species. In this group, there are genes involved in gly-
colysis (\textit{aldE}, \textit{pgk}, and \textit{eno}), formate fermentation (\textit{focA}), and
glucose and mannitol utilization (\textit{ptsG}, \textit{ptsHI-crr}, and
\textit{mltAD}).

**Lineage-specific HexR-regulated Genes**—The rest of the pre-
dicted target genes belong to a group of lineage-specific regulon
members (Table 2). At most, this group includes genes with
candidate HexR-binding sites that are conserved in at least two
species within the same lineage but that are not conserved out-
side of this lineage. This group also includes the
\textit{Colwellia psychrerythraea} fructose-biphosphate aldolase gene, \textit{fba}, which
is preceded by a strong HexR-binding site. The largest set of lin-
eage-specific HexR targets was detected in the Shewanella species, and it includes genes from the central carbohydrate metabolism (gnd, phk, and adhB), nucleoside/deoxyribonucleoside utilization (deoABD, nupC, and cdd), respiratory chain (npr operon), and glycine utilization (gcvTHP). The second largest pool of predicted lineage-specific HexR-regulated genes was detected in Vibrio species; it includes glycogen metabolism genes, a nitrite reductase, a peptidase, and a lactate permease. Extended HexR regulons in other lineages contain genes involved in utilization of galactosides and glycerol (mgl and glpT), glycolysis (glpN), the tricarboxylic acid cycle (gltA), lactate, and acetate fermentation (ldhA and ackA-pta).

**Experimental Validation of HexR Regulon in S. oneidensis MR-1**

**Dual Mode of HexR Regulation**—The reconstructed HexR regulon in *S. oneidensis* MR-1 contains 30 genes (organized in 15 operons) that are involved in various metabolic pathways (Fig. 1B). These include two opposite central carbon metabolism pathways, the glycolytic (ED) pathway for hexose catabolism (zwf-pgl-edd-edd, gapA, and pykA) and the gluconeogenic pathway for glucose biosynthesis (ppsA and gapB). This observation suggests that HexR may have an opposite regulatory effect on the target gene expression, similar to the Cra dual repressor/activator control of carbon metabolism in *E. coli* (4). To reveal a negative or positive mode of HexR regulation in *Shewanella* we used several complementary approaches as follows: (i) the comparative analysis of upstream promoter sequences in multiple *Shewanella* genomes; (ii) the analysis of correlations of gene expression profiles in the compendium of microarray data available for *S. oneidensis* MR-1; and (iii) RT-PCR analysis of regulon gene expression in hexR knock-out mutant (see below).

For each predicted HexR-regulated gene in *S. oneidensis* MR-1, DNA upstream regions of orthologous genes from other *Shewanella* genomes were aligned, and candidate HexR-bind sites and potential promoter elements (the −35 and −10 promoter boxes) were located (supplemental Fig. S2). For 9 genes, namely zwf, pykA, gapA, gnd, nqrA, deoA, cdd, phk, and mcp, HexR operators either overlap with or are located downstream of the putative conserved promoter elements, suggesting that HexR is a repressor of these genes. In contrast, five other genes (ppsA, gapB, gcvT, tal, and nupC) have HexR operators located upstream of predicted promoters (distance between the center of HexR site and the 5' position of the −35 promoter box equal to either 29, 21, or 14 nucleotides), suggesting that HexR may be acting as an activator.

To evaluate pairwise correlations in expression of all HexR-regulated genes, we have computed the Pearson correlation between each pair of genes in the *S. oneidensis* MR-1 genome based on the expression of each gene in ~200 microarray experiments available in the MicrobesOnline database (supplemental Fig. S3). This analysis allowed us to identify subregulons that have different expression patterns. There is a strong correlation within the first group of zwf, gapA, pykA, and tal genes and the second group of phk, deoA, cdd, and nqrA genes, although correlation between these two groups is also significant. The third group of highly correlated genes gapB, ppsA, and gcvT shows no correlation with the genes from the first two groups. Indeed, the expression data previously obtained for *S. oneidensis* MR-1 grown on various carbon sources (34) confirm up-regulation of the deoA, zwf, phk, nupC, gapA genes during growth on inosine, whereas ppsA, gapB, and gcvT genes were down-regulated in these conditions compared with the growth on lactate. The observed different patterns of gene expression

### Table 2

| Taxonomic group(s) | Target gene(s) | No. | Functional role |
|-------------------|---------------|-----|----------------|
| Shewanellaceae (16) | phk | 16 | Phosphoketolase |
|                    | gnd | 14 | 6-Phosphogluconate dehydrogenase |
|                    | nqrABCDEF | 15 | NADH:ubiquinone oxidoreductase |
|                    | deoABD | 14 | Deoxynucleoside utilization |
|                    | nupC | 15 | Nucleoside transporter |
|                    | cdd | 10 | Cytidine deaminase |
|                    | gcvTHP | 12 | Glycine cleavage |
|                    | adhB | 8 | Alcohol dehydrogenase II |
|                    | mcp | 9 | Chemotaxis protein |
|                          | SO1118 | 10 | Hypothetical protein |
|                          | mglABAC | 3 | Galactoside utilization |
|                          | gltP | 2 | Glycerol utilization |
|                          | ldhA | 3 | Lactate dehydrogenase |
|                          | ackA-pta | 4 | Acetate kinase |
|                          | gntA | 2 | Citrate synthase |
|                          | mgsA | 2 | Methylglyoxal synthase |
|                          | PF00248 | 2 | Putative aldo/keto reductase |
|                          | fba | 1 | Fructose-bisphosphate aldolase |
|                          | gldP | 2 | Glycerol phosphorylase |
|                          | cpsA | 2 | Capsular polysaccharide synthesis enzyme |
|                          | ybfA | 10 | Putative exported protein |
|                          | pepD | 9 | Aminoacyl-histidine dipeptidase |
|                          | nitB | 7 | Nitrite reductase (NADPH) |
|                          | glyX | 6 | Glycogen debranching enzyme |
|                          | glgCA | 5 | Glycogen synthase, glucose-1-phosphate adenlylytransferase |
|                          | lctP | 2 | i-Lactate permease |
|                          | ypeW | 8 | Putative inner membrane protein |
|                          | gapN | 2 | Putative glyceraldehyde-3P dehydrogenase |
|                          | glRS | 7 | Glucose uptake regulatory system |

* The number of genomes in the analyzed taxonomic group is indicated in parentheses.

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in the HexR regulon and the sequence analysis of promoter gene regions in S. oneidensis MR-1 suggest that HexR is a dual mode regulator that can be either repressor or activator of genes expression depending on the relative position of its operators and promoters.

HexR Binds Its Cognate DNA Sites in Vitro—To test the ability of HexR to specifically bind to the predicted DNA sites and to assess effectors, hexR (SO2490) from S. oneidensis, MR-1 was cloned and overexpressed in E. coli. The Smt3-His6-tagged recombinant protein HexR was purified by Ni²⁺/H₁₀₀₀₁-chelating chromatography, followed by gel filtration as described under “Experimental Procedures.” Electrophoretic mobility shift assay (EMSA) and FPA were used to test specific binding of the purified HexR protein to its predicted operator sites in upstream regions of 15 genes in S. oneidensis MR-1 (Table 3).

Binding of purified HexR to the SO2489 dsDNA fragment containing the predicted HexR-binding site resulted in the reduced electrophoretic mobility of the DNA fragment in a concentration-dependent manner (supplemental Fig. S4). Of the tested intermediary metabolites associated with the ED pathway (2 mM KDPG, 6-phosphoglucose, phosphoenolpyruvate, or 6-phosphoglucunate), only KDPG demonstrated significant suppression of the HexR-DNA binding-mediated shift. These findings are in agreement with the previous results obtained for the P. putida HexR regulator, which binds DNA in the absence of an effector, whereas KDPG induces the HexR-DNA complex dissociation (8). Specific binding at 0.5 M HexR was also confirmed for all other tested DNA fragments (supplemental Table S1) with the single exception of the SO0779 dsDNA fragment (supplemental Fig. S4 B). The latter fragment did not show a clear shift in the EMSA experiments but was confirmed to bind HexR using the FPA assay (see below).

The FPA binding assay was used to assess the specific binding of eight predicted HexR-binding sites in S. oneidensis with increasing concentrations of the HexR protein (Fig. 3). All tested DNA fragments demonstrated the HexR concentration-dependent increase of fluorescence polarization, confirming specific interactions between HexR and DNA. The apparent $K_d$
values for HexR protein interacting with the tested DNA fragments were in the range of 28–71 nM (Table 3). As a negative control, we assessed interaction between a different S. oneidensis transcription factor (NagR or SO3516) with the SO2489 DNA fragment, and the HexR regulator with the SO3507 DNA fragment containing the previously confirmed NagR-binding site (12), and in both experiments no significant change of fluorescence polarization was detected. These results confirm that HexR is a KDPG-responsive regulator that binds specifically to the predicted operator sites in S. oneidensis MR-1.

**HexR Affects Expression of Its Target Genes in Vivo**—To validate the predicted mode of HexR regulation on gene expression in vivo, a ΔhexR targeted deletion mutant strain of S. oneidensis MR-1 was constructed, and the relative transcript levels of the predicted HexR target genes analyzed by quantitative RT-PCR (Table 3). Relative mRNA levels of nine genes were elevated more than 1.5-fold in the ΔhexR mutant compared with the WT strain when grown in the minimal medium supplied with either glycerate or inosine. The most prominent effect of hexR mutation was observed for the zwf, pykA, nqrA, phk, deoA, and gapA genes, suggesting that HexR represses expression of these genes. The cdd and mcp genes were up-regulated 2–3-fold in ΔhexR strain in the media supplied with glycerate, whereas gnd was up-regulated near 2-fold in the cells grown on inosine. Expression of the tal gene was not significantly affected by hexR mutation in both conditions. In contrast, the ppsA, gapB, and nupC genes were significantly down-regulated in the ΔhexR strain. For instance, expression of the ppsA gene was decreased 9- and 17-fold in the cells grown on inosine and glycerate, respectively. Finally, expression of gcvT was down-regulated 3-fold in the ΔhexR strain when the cells were grown on glycerate but was up-regulated 2-fold in the inosine-supplied cells. In our previous study of metabolic regulators in Shewanella spp. (12), we have identified several regulatory motifs in the upstream region of the gcvTHP operon (supplemental Fig. S2), which is presumably regulated by several factors, including the HexR activator, the glycine-responsive activator GcvA, and the previously unknown regulator of purine metabolism operated by binding to PUR-boxes. These overlapping regulatory interactions could possibly explain the observed differences in the expression patterns of the gcvH genes in ΔhexR strain grown on either glycerate or inosine, as the purine nucleoside could potentially repress the gcv operon via a PUR-box operator. The observed differences in the regulation of gnd and mcp promoters in strains grown on different carbon sources could be explained by possible involvement in their regulation of other still unknown regulatory mechanisms that can differentiate the tested carbon sources. These observations confirm the predicted negative mode of HexR regulation for the zwf, pykA, nqrA, phk, deoA, gapA, gnd, cdd, and mcp genes and its positive mode of action on the ppsA, gapB, nupC, and gcvT genes.

**Phenotype Characterization of S. oneidensis ΔhexR Mutant**—The previous phenotypic and genomic analyses suggest that S. oneidensis can use GlcNAC, glycerate, inosine, lactate, and pyruvate as the sole carbon source (13, 34, 35). Because of the absence of phosphofructokinase in Shewanella spp., utilization of GlcNAC proceeds through glucose 6-phosphate, which is catabolized via the HexR-regulated ED pathway (Fig. 1B). Utilization of inosine requires the HexR-regulated nupC and deo-ABD genes that mediate nucleoside uptake, release of ribose base, and its further utilization via the pentose phosphate pathway. The catabolic pathways produce pyruvate, which is further utilized via the fermentation/respiration routes to gain energy and the gluconeogenesis for biosynthetic needs. To determine whether the loss of HexR function impairs the utilization of carbon sources, the S. oneidensis MR-1 WT and ΔhexR mutant strains were tested for the ability to grow on the above five carbon sources. The hexR mutant was unable to grow on lactate or pyruvate but grew similar to the WT on GlcNAC (Fig. 4) or inosine (data not shown). An enhanced growth on glycerate was observed for the ΔhexR mutant compared with the WT strain (Fig. 4). The inability of the S. oneidensis ΔhexR strain to grow on lactate as a sole carbon source confirms that HexR is an important transcriptional activator of the gluconeogenic gene ppsA, whose activity is known to be essential for the growth of E. coli on lactate (36). Therefore, based on a combination of genotypic analysis, metabolic reconstruction (31), and physiological data, we hypothesized that HexR controls the main gluconeogenic flux from lactate via activation of PpsA. Disruption of this flux in ΔhexR mutant might be the main cause of its inability to grow on lactate as a single carbon source. However, the flux from lactate through the tricarboxylic acid cycle appears to be HexR-independent, and thus, it might continue in its role in energy production even in the ΔhexR mutant (in the presence of an additional carbon source, see below).

**Metabolic Flux Analysis of S. oneidensis ΔhexR Mutant**—To test this hypothesis, the intracellular carbon fluxes in the WT and the ΔhexR mutant strains were quantified by using the 13C-based metabolic flux analysis that relies on the [U-13C]lactate labeling and GC-MS analysis of the mass isotopomer pattern in proteinogenic amino acids (supplemental Table S4). From the labeling patterns of the respective amino acids, the origins of phosphoenoxyruvate were quantitatively determined (Fig. 5). Phosphoenolpyruvate can be derived from pyruvate via phosphoenolpyruvate synthase encoded by ppsA, from oxaloacetate via phosphoenolpyruvate carboxykinase, or from glycerate through the glycolytic conversion of 3-phosphoglycerate to phosphoenolpyruvate. In the S. oneidensis MR-1 WT strain grown on the mixture of lactate and glycerate, more than 40% of phosphoenolpyruvate originated from the phosphoenolpyruvate synthase reaction (Fig. 5). In contrast, the phosphoenolpyruvate synthase flux was absent, and the majority of phosphoenolpyruvate was derived from glycerate in the ΔhexR mutant strain. A similar result was also found for the ΔppsA mutant strain (supplemental Table S4). These results indicate that the ppsA-encoding phosphoenolpyruvate synthase is largely inactive in vivo in the ΔhexR mutant. Moreover, the HexR deficiency also resulted in significant changes in central metabolic fluxes. Compared with the WT, a significant increase in the uptake fluxes of lactate and glycerate was observed for the ΔhexR mutant. This mutant also exhibited much higher fluxes of secretion of acetate and pyruvate.
DISCUSSION

Transcriptional control of metabolic pathways for carbohydrate utilization in bacteria is mediated by a variety of TFs from different protein families (e.g. AraC, LacI, GntR, DeoR, and ROK). Peripheral sugar catabolic pathways are usually controlled by committed transcription factors with presumably local regulons (e.g. the GlcNAc utilization pathway is regulated by NagR repressor in Shewanella) (13), whereas central glycolytic pathways are regulated by global TFs (e.g. the EMP pathway is under control of Crp and FruR in E. coli) (1). Previous studies show that P. putida HexR is a KDPG-responsive transcriptional regulator that negatively controls the edd-glk-gltRS, zwf-pgl-eda, and gap genes involved in the ED and EMP pathways of glucose utilization (8–11). Here, we performed the comparative genomics reconstruction of HexR regulons in 87 species of Proteobacteria by combining the identification of HexR-binding sites with the analysis of functional genomic context. We report a high variability in gene content of the reconstructed HexR regulons in different taxa, although the cognate DNA motifs of HexR orthologs share a common palindromic consensus sequence TGTA-N7-TACA. The combined bioinformatics, in vitro and in vivo characterization of the extended HexR regulon in Shewanella spp., revealed that HexR functions as both a repressor and activator on different promoters involved in the catabolic and gluconeogenic pathways, respectively. The discovered dual mode of action and the functional context of target genes indicate that HexR is a novel pleiotropic regulator of the central carbon metabolism in Proteobacteria. In Shewanella, HexR usually controls various genes from the central carbon metabolism, as well as the deoxyribose and glycine utilization.

HexR belongs to the RpiR family of TFs that are characterized by an N-terminal DNA binding helix-turn-helix domain (HTH_6 or PF01418 in Pfam database) and a C-terminal sugar phosphate binding domain (SIS or PF01380) (37). The presence of the SIS effector binding domain in regulators from the RpiR family correlates with their known ability to bind various sugar phosphates, including KDPG for HexR in P. putida (8, 10), allose 6-phosphate for RpiR/AlsR in E. coli (38), N-acetylmuramic acid 6-phosphate for MurR in E. coli (39), and glucos-
amine 6-phosphate for SiaR in *Haemophilus influenzae* (40). Multiple alignment of HexR proteins revealed that among the residues that were previously predicted to be more important for the recognition of DNA in *P. putida* HexR (according to a homology-based three-dimensional model of HexR (8)), only Arg-54 is absolutely conserved in orthologs; Glu-49 and Arg-57 are present in more than 90% of orthologs, whereas Gln-43 and Lys-46 are present only in HexR orthologs in *Pseudomonas* spp. These observations correlate with the relative conservation of the identified HexR-binding DNA motifs. Interestingly, the same residues are retained in a HexR paralog translationally fused to a glucokinase domain in *Burkholderia* spp., suggesting that this chimeric protein could function both as a kinase and a transcriptional regulator. Two residues likely involved in effector recognition in *P. putida* HexR, Ser-140 and Ser-184 (8), were found absolutely conserved in all HexR orthologs, suggesting that its effector molecule KDPG validated in *P. putida* (8) and *S. oneidensis* (this study) is conserved in all HexR orthologs.

Because of the absence of 6-phosphofructokinase, the ED pathway is an essential sugar catabolic route, and KDPG is its key intermediate in *Shewanella*, as well as in *Pseudomonas, Burkholderia,Ralstonia* and some other Proteobacteria. In this work, we demonstrated that the HexR protein in *Shewanella* is a KDPG-responsive repressor/activator of the central carbon metabolism (Fig. 1). The proposed model of HexR-dependent control in *Shewanella* is presented below. In the presence of carbohydrates (hexoses or pentoses), KDPG levels in the cell increase due to the consecutive action of peripheral and central catabolic pathways. Binding of KDPG to the HexR SIS domain promotes dissociation of HexR-DNA complexes, leading to de-repression of transcription of the central glycolytic genes (*zwf-pgl-edd-eda*, *gapA*, *pykA*, *gnd*, and *phk*) and de-activation of the *ppsA* and *gapB* genes involved in the gluconeogenesis. In contrast, when carbohydrates are limited in the medium (e.g. when the cell is growing on lactate or pyruvate as the sole carbon source), the KDPG level is decreased; and HexR binding to its operator sites promotes
repression of the glycolytic genes and activation of the gluconeogenic genes (Fig. 6).

Lactate is a major source of carbon and energy for Shewanella, a diverse group of dissimilatory metal-reducing bacteria commonly found in aquatic and sedimentary environments (35). Based on the combination of regulatory and phenotype analysis of *S. oneidensis* wild-type and ΔhexR mutant (Fig. 4), we concluded that HexR is a master regulator of the gluconeogenic flux from pyruvate (and lactate). Thus, when lactate is present as a single carbon source, it is used for both energy production (via the tricarboxylic acid cycle) and biosynthesis (via gluconeogenesis). This is achieved via a low level of KDPG leading to transcriptional activation of *ppsA* and down-regulation of *pykA* (to avoid futile cycling). When lactate is present simultaneously with other carbon sources such as GlcNAc, most of the lactate would route to the tricarboxylic acid cycle for energy, whereas biosynthetic needs are supported by GlcNAc. This is achieved via accumulation of KDPG, which interacts with HexR, resulting in de-activation of *ppsA* and de-repression of *pykA*. According to this model, the pyruvate kinase PykA up-regulation would promote disposal of excess glycolytic substrate via an elevated flux from phosphoenolpyruvate to pyruvate. This situation is modeled by deletion of *HexR* or by deletion of *ppsA* (Fig. 5).

The HexR regulon in *Shewanella* can be considered as a partial functional replacement of the Enterobacterial Cra (FruR) regulon that plays a pleiotropic role, modulating the direction of carbon flow through the different metabolic pathways of energy metabolism. The catabolite repressor/activator Cra in *E. coli* binds DNA operators in the absence of fructose 1-phosphate or fructose 1,6-bisphosphate and is inactivated upon the interaction with the effectors (3). When bound to its targets, it acts as an activator of gluconeogenic (*ppsA* and *pckA*) and glyoxylate shunt (aceBA) genes and a repressor of glycolytic genes (pykF, gapA, edd-edA, eno, pgk, and *pykA*) (41). Thus, both HexR and Cra are dual transcriptional regulators that mediate cAMP-independent catabolite control in response to key catabolite intermediates. *Shewanella* spp. does not have Cra and are not able to grow on fructose (13). The Cra (FruR) regulons reconstructed in other γ-proteobacteria (Aeromonadales, Pseudomonadales, Psychromonadaceae, and Vibrionales) are implicated in the local control of the fructose utilization operon (5). The HexR regulons are local (regulate 1–2 operons) in some lineages (e.g. in Enterobacteriales, *Ralstonia*, and *Burkholderia*) and global (control 15–20 operons) in other lineages (e.g. in *Shewanella*, Aeromonadales, Psychromonadaceae, and Vibrionales). For instance, in *E. coli* HexR has only two predicted target genes (zwf and ybfA), whereas in *Photobacterium profundum* it controls near 20 operons implicated in several central metabolic pathways. These observations suggest that regulatory networks for the central carbon metabolism are very flexible in Proteobacteria. Global rewiring of these regulatory networks includes multiple regulon expansion and reduction events.

Interestingly, the KDG-responsive KdgR regulon negatively controlling the pectin catabolism in plant pathogenic *Erwinia* is largely extended in comparison with other enterobacteria and include the positively regulated gene *ppsA* (42). Therefore, the *ppsA* gene is activated by at least three dual-mode catabolic regulators (HexR, FruR, and KdgR) in three lineages of γ-proteobacteria (*Shewanella*, *Escherichia*, and *Erwinia*, respectively).

**Concluding Remarks**—This study provided a comprehensive bioinformatic analysis of the HexR regulon that constitutes a pleiotropic system of transcriptional regulation of central carbohydrate metabolism in several groups of Proteobacteria. The key conjectures delivered by this analysis, the global regulatory effect and dual mode of HexR-dependent regulation, were experimentally confirmed in the *S. oneidensis* model providing additional support for the reconstruction of the entire HexR regulon in Proteobacteria. This study demonstrates the value of comparative genomics supported by focused validation experiments for the *ab initio* reconstruction regulatory networks in large sets of previously unexplored biological species.

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