Genetic Basis of Chromate Adaptation and the Role of the Pre-existing Genetic Divergence during an Experimental Evolution Study with *Desulfovibrio vulgaris* Populations

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ABSTRACT Hexavalent chromium [Cr(VI)] is a common environmental pollutant. However, little is known about the genetic basis of microbial evolution under Cr(VI) stress and the influence of the prior evolution histories on the subsequent evolution under Cr(VI) stress. In this study, *Desulfovibrio vulgaris* Hildenborough (DvH), a model sulfate-reducing bacterium, was experimentally evolved for 600 generations. By evolving the replicate populations of three genetically diverse DvH clones, including ancestor (AN, without prior experimental evolution history), non-stress-evolved EC3-10, and salt stress-evolved ES9-11, the contributions of adaptation, chance, and pre-existing genetic divergence to the evolution under Cr(VI) stress were able to be dissected. Significantly decreased lag phases under Cr(VI) stress were observed in most evolved populations, while increased Cr(VI) reduction rates were primarily observed in populations evolved from EC3-10 and ES9-11. The pre-existing genetic divergence in the starting clones showed strong influences on the changes in lag phases, growth rates, and Cr(VI) reduction rates. Additionally, the genomic mutation spectra in populations evolved from different starting clones were significantly different. A total of 14 newly mutated genes obtained mutations in at least two evolved populations, suggesting their importance in Cr(VI) adaptation. An in-frame deletion mutation of one of these genes, the chromate transporter gene DVU0426, demonstrated that it played an important role in Cr(VI) tolerance. Overall, our study identified potential key functional genes for Cr(VI) tolerance and demonstrated the important role of pre-existing genetic divergence in evolution under Cr(VI) stress conditions.

IMPORTANCE Chromium is one of the most common heavy metal pollutants of soil and groundwater. The potential of *Desulfovibrio vulgaris* Hildenborough in heavy metal bioremediation such as Cr(VI) reduction was reported previously; however, experimental evidence of key functional genes involved in Cr(VI) resistance are largely unknown. Given the genetic divergence of microbial populations in nature, knowledge on how this divergence affects the microbial adaptation to a new environment such as Cr(VI) stress is very limited. Taking advantage of our previous study, three groups of genetically diverse *D. vulgaris* Hildenborough populations with or without prior experimental evolution histories were propagated under Cr(VI) stress for 600 generations. Whole-population genome resequencing of the evolved populations

revealed the genomic changes underlying the improved Cr(VI) tolerance. The strong influence of the pre-existing genetic divergence in the starting clones on evolution under Cr(VI) stress conditions was demonstrated at both phenotypic and genetic levels.

KEYWORDS chromate stress, *Desulfovibrio vulgaris*, experimental evolution, genetic background

Chromium is a very common environmental pollutant and imposes an increasing threat to the environment, public health, and the economy (1, 2). Industrial applications, such as electroplating, leather tanning, wood preservation, and mine tailings, are the main causes of chromium contamination (3, 4). It was reported that over 50% of the 170 U.S. Department of Energy (DOE) sites have been contaminated with chromium, which originated from nuclear fuel production waste, nuclear research, and nuclear reactor operations at U.S. DOE facilities (5–8). Chromium exists predominantly in a trivalent state [Cr(III)] and hexavalent state [Cr(VI)] in the environment (9). Cr(VI) is well documented as a highly water-soluble carcinogen and mutagen (10–12). It has been reported that Cr(VI) actively crosses biological membranes in several bacterial species via sulfate transport pathways owing to the chemical structural similarity between CrO_4^{2-} and SO_4^{2-} (13). In contrast, Cr(III) is relatively insoluble and is considered less toxic than Cr(VI) (14). Conventional chromium detoxification methods, such as chemical reduction, anion exchange, and physical adsorption, require large amounts of chemicals and energy (15, 16). In comparison, biological reduction of Cr(VI) may be a promising alternative for detoxification because of its low chemical and energy consumption and high conversion efficiency (17, 18).

Desulfovibrio vulgaris Hildenborough (DvH) has been extensively studied as a model sulfate-reducing bacterium (SRB) and plays an important role in biogeochemical cycling and potentially the remediation of heavy metal contamination (19–21). SRB are commonly found in environments contaminated with heavy metals and other pollutants (e.g., high concentrations of NaCl and nitrate) that are lethal to many other microorganisms (22, 23). Effective reduction of Cr(VI) and other heavy metals by SRB was first reported decades ago (19, 20, 24, 25). It has been proposed that SRB-mediated heavy metal reduction includes an indirect chemical reduction involving H_2S produced from sulfate reduction and an enzymatic process involving various molecular forms of *c*-type cytochromes, hydrogenases, and ferredoxins (19, 20, 23, 26), while the biological reduction of heavy metals such as Cr(VI) or U(VI) cannot generate energy for cell growth (20, 27). An important mechanism of Cr(VI) resistance in bacteria is the efflux of chromate conferred by transporters, like the ChrA protein (28). ChrA belongs to the chromate ion transporter (CHR) superfamily and carries out efflux of chromate from the cytoplasm coupled with proton motive force (29). The DvH genome has two annotated chromate transporter genes, with one gene, DVU0426, present on the chromosome and another gene, DVUA0093, on the native plasmid. These chromate transporters may act as chromate efflux pumps (30–32). Other genes, such as the hypothetical gene DVUA0095 on the native plasmid, may play a role in the Cr(VI) stress response, since its deletion mutant showed increased Cr(VI) sensitivity (33). A recent study showed that Cr(VI) reduction and physiological toxicity in DvH were greatly impacted by temperature and resource (donor/acceptor) ratio (34). All these results suggested the complexity of Cr(VI) resistance mechanisms. Identification of the key functional genes involved in Cr(VI) resistance is crucial for understanding the significance of DvH in bioremediation of environmental Cr(VI) contamination.

Experimental evolution has been considered an excellent approach to uncover the molecular mechanisms of complex traits. Next-generation whole-genome sequencing of the experimentally evolved microorganisms has been widely used to uncover the correlation between genomic mutations and the phenotypic changes, which greatly improved our understanding of the genetic basis underlying the desired traits as well

as fundamental evolutionary processes (35). In DvH, this approach has been successfully applied to study adaptation mechanisms under salt (NaCl) stress, which is also present in several DOE sites contaminated with toxic metals. The genomic changes associated with the markedly increased NaCl tolerance in the evolved populations were revealed by whole-genome sequencing and confirmed by site-directed mutagenesis (36–38). Therefore, experimental evolution of DvH under Cr(VI) stress and characterization of the evolved populations would allow us to uncover key genes involved in Cr(VI) resistance.

Adaptation, chance, and history are considered the main driving forces in evolution (39). Adaptation leads to the survival of the best-adapted organisms in populations via natural selection. Chance, including mutation and genetic drift (40), is important because mutations arise at random and can be lost due to genetic drift (41). History can be an important factor, since certain prior genetic changes either constrain or promote the evolutionary outcomes in the subsequent evolution in new environments (42). Experimental evolution has been a powerful approach to address the roles of these three major driving forces in evolution by two-phase evolution experiments. For instance, Travisano et al. reported such a test with experimental evolution of *Escherichia coli* (41). First, replicate populations from different ancestral genotypes were generated and propagated in identical environments. Then, specific traits of the ancestral and evolved populations were measured to assess the roles of adaptation, chance, and history. Similar experiments have been performed in many other organisms (43). Given the existence of multiple stressors in heavy metal-contaminated field sites, very little is known about the influence of genetic divergence derived from evolution history under one stress (e.g., high NaCl concentration) on the adaptation of DvH to another stress, such as Cr(VI) stress. With a similar experimental setup, by propagating genetically diverse DvH populations under Cr(VI) stress and measurement of the specific traits of the ancestors and the evolved populations, the roles of the pre-existing genetic divergence as well as adaption and chance in DvH evolution under Cr(VI) stress could be evaluated (see Fig. S1 in the supplemental material).

In this study, we aimed to address the following questions. (i) What are the genetic changes and key mutations underlying the improved Cr(VI) tolerance? (ii) What are the contributions of adaptation, chance, and pre-existing genetic divergence to the evolution of DvH under Cr(VI) stress? To answer these questions, three genetically diverse DvH clones generated from our previous studies, including ancestor (AN; without experimental evolution history), EC3-10 (with 1,200 generations of evolutionary history under nonstress conditions), and ES9-11 (with 1,200 generations of evolutionary history under salt stress) (36), were evolved under Cr(VI) stress for 600 generations with six replicates in each group. The pre-existing genetic divergence had significant influence on the phenotypic evolution of characteristics such as lag phase, growth rate, and Cr(VI) reduction rate. In addition, the genomic changes underlying the phenotypic changes and the strong influence of starting genotypes on genetic evolution were uncovered by whole-population sequencing of the evolved populations. Characterization of mutants demonstrated the important role of the gene DVU0426 in Cr(VI) tolerance.

RESULTS

Increased Cr(VI) resistance and Cr(VI) reduction ability in the starting clone ES9-11. We initiated our study with three starting clones, ancestor (AN), EC3-10, and ES9-11. AN was a clonal isolate of DvH originally from ATCC 29579. EC3-10 and ES9-11 were clonal isolates derived from DvH populations EC3 and ES9, which had been evolved under nonstress (LS4D medium) or salt stress (LS4D plus 100 mM NaCl) conditions, respectively, for 1,200 generations (Fig. 1). EC3-10 and ES9-11 were the representatives of the best salt tolerance phenotypes of populations EC3 and EC9. Both EC3-10 and ES9-11 gained significantly increased salt tolerance (increased growth rate, increased final biomass, and shortened lag phase) compared with AN, with ES9-11 showing better salt tolerance than EC3-10. Improved salt tolerance in EC3-10 might be
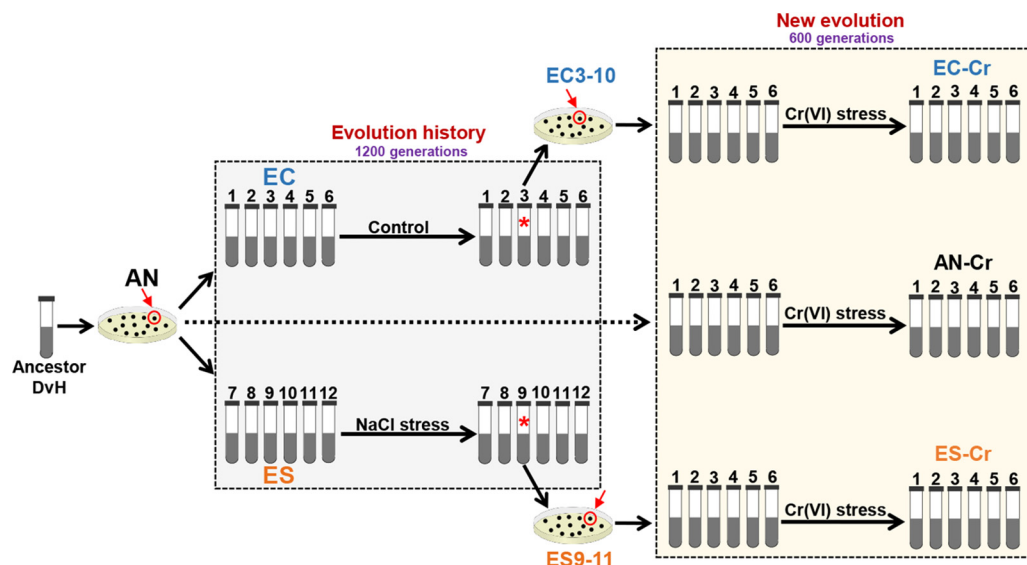
FIG 1 Schematic representation of the experimental design. Laboratory experimental evolution of DvH populations under Cr(VI) stress was initiated from three starting clones. The ancestor clone (AN) had no experimental evolution history. EC3-10 and ES9-11 were isolated from populations that had been propagated from AN under control or salt stress conditions for 1,200 generations, respectively (evolution history). Six replicates from each starting clone were evolved under Cr(VI) stress for 600 generations (new evolution).

due to the presence of about 210 mM total Na$^+$ in LS4D medium. Under control conditions, ES9-11 also showed a significantly increased growth rate, increased final biomass, and shortened lag phase compared with AN or EC3-10 (36). In addition, both EC3-10 and ES9-11 showed higher growth rates under a novel stress condition (elevation of temperature to 41°C) (44). To assess whether the evolutionary history of EC3-10 and EC9-11 affected their Cr(VI) resistance, the growth phenotypes and Cr(VI) reduction capacities of AN, EC3-10, and ES9-11 were measured. Under Cr(VI) stress, ES9-11 had a significantly ($P < 0.001$) shorter lag phase than EC3-10 and AN, while the maximum growth rate of ES9-11 was significantly ($P < 0.01$) decreased compared to that of EC3-10 and AN (Fig. 2a and b). In addition, ES9-11 had a significantly higher Cr(VI) reduction rate than EC3-10 ($P < 0.01$) and AN ($P < 0.05$), while the Cr(VI) reduction rates of EC3-10 and AN were similar (Fig. 2c). These data demonstrated that salt-evolved ES9-11 had increased Cr(VI) resistance (significantly decreased lag phase) and Cr(VI) reduction ability compared to both control-evolved EC3-10 and AN.

Improved Cr(VI) resistance and Cr(VI) reduction ability in Cr(VI) stress-evolved populations. Six replicate populations were initiated from each of the starting clones, and these replicate populations were propagated under Cr(VI) stress for 600 generations (Fig. 1). To investigate whether the phenotypic and genetic differences in the starting clones affected the adaptation to Cr(VI) stress, growth phenotypes and Cr(VI) reduction capacities of the Cr(VI) stress-evolved populations AN-Cr-1 to -6, EC-Cr-1 to -6, and ES-Cr-1 to -6 were assessed. Under control conditions, most Cr(VI) stress-evolved populations (four AN-Cr, five EC-Cr, and two ES-Cr populations) grew better, with significantly ($P < 0.05$) decreased lag phases or increased growth rates compared to their starting clones (Fig. S2 and S3). Under the Cr(VI) stress condition, most Cr(VI) stress-evolved populations showed significantly ($P < 0.05$) shortened lag phases compared to their starting clones (Fig. 3a; Fig. S2). The grand mean lag phase of the 18 evolved populations was significantly decreased compared to that of the ancestors (*t* test, $P = 0.002$), indicating the strong influence of adaptation. The contributions of adaptation, chance, and the pre-existing genetic divergence to the changes in lag phases were 19.3%, 26.3% ($F = 67.121$; df = 15 and 36; $P < 0.001$), and 54.4% ($F = 4.277$; df = 2 and 15; $P = 0.032$), respectively (Fig. 3b). The growth rates of most evolved populations were unchanged or decreased (Fig. 3c; Fig. S2), resulting in an unchanged grand mean
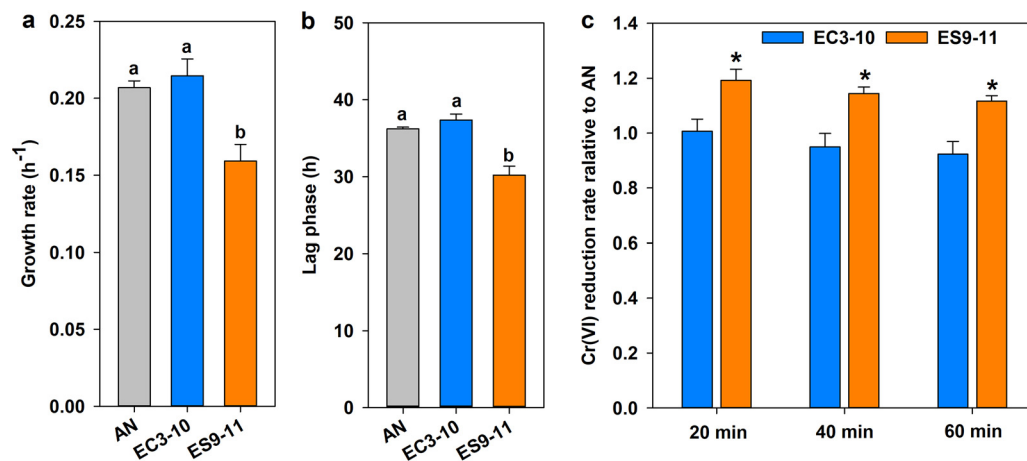
FIG 2 Improved Cr(VI) tolerance and Cr(VI) reduction rate in salt (NaCl) stress-evolved clone ES9-11 compared to the control-evolved EC3-10 clone and AN. The growth rates (a) and lag phases (b) of the three starting clones in LS4D medium supplemented with 0.16 mM Cr(VI) are shown (*t* test, $P < 0.01$). (c) Cr(VI) reduction rates of EC3-10 and ES9-11 relative to AN at 20 min, 40 min, and 60 min after addition of Cr(VI) in the washed-cell experiments. *, $P < 0.05$ (*t* test). Error bars represent standard deviations for three biological replicates.

growth rate of the 18 evolved populations (*t* test, $P = 0.137$). In the evolution of growth rates, the pre-existing genetic divergence played a dominant role (83.3%, $F = 24.976$; df = 2 and 15; $P < 0.001$), and chance played a minor role (16.7%, $F = 4.038$; df = 15 and 36; $P < 0.001$) (Fig. 3d). In terms of Cr(VI)-reduction capability (Fig. 3e; Fig. S4), the grand mean Cr(VI) reduction rates significantly increased in both the EC-Cr group (*t* test, $P = 0.035$) and the ES-Cr group (*t* test, $P = 0.002$) compared to their starting clones. However, the grand mean Cr(VI) reduction rate of 18 evolved populations was not significantly changed (*t* test, $P = 0.361$). Changes in Cr(VI) reduction rates were contributed by the pre-existing genetic divergence (81.2%, $F = 18.604$; df = 2 and 15; $P < 0.001$) and chance (18.8%, $F = 13.783$; df = 15 and 36; $P < 0.001$) (Fig. 3f). Together, these results demonstrated the strong influences of the pre-existing genetic divergence on the changes in lag phases, growth rates, and Cr(VI) reduction capability during adaptation to Cr(VI) stress.

Strong influences of the genetic backgrounds in starting clones on genomic mutation spectra in Cr(VI) stress-evolved populations. To identify the genetic changes obtained during Cr(VI) evolution and evaluate the influence of the diverse genotypes in the starting clones, we sequenced the genomes of all 18 Cr(VI) stress-evolved populations. An average coverage depth of 340× was achieved with Illumina sequencing. Mutations with frequencies above 10% were selected for further analysis. Our previous study demonstrated that there were 11 polymorphic loci in the AN clone compared to the NCBI reference DvH sequence NC_002937.3, 14 mutations (six polymorphism-derived mutations and eight new mutations) in non-stress-evolved clone EC3-10, and 11 mutations (five polymorphism-derived mutations and six new mutations) in salt stress-evolved clone ES9-11 (37). These mutations were defined as pre-existing mutations in the starting clones in this study and excluded from the following statistical analysis. Analysis of the mutations in Cr(VI)-stress evolved populations demonstrated that the pre-existing mutations in starting clones EC3-10 and ES9-11 remained stable in all EC-Cr and ES-Cr populations. In addition, all AN-Cr populations had five polymorphism-derived mutations, which were the same as the five polymorphism-derived mutations in the salt stress-evolved population ES9, suggesting that the selection of these pre-existing polymorphic loci might be common under stress conditions. The mutations acquired during Cr(VI) evolution were defined as new mutations (146 mutations in all 18 populations) (Table S1). The number of new mutations in each evolved population varied from 4 to 20, and the average numbers of mutations in the AN-Cr, EC-Cr, and ES-Cr populations were 8, 10, and 7, respectively (Fig. 4a). In these new mutations, missense mutations and indels were the most common variant types.
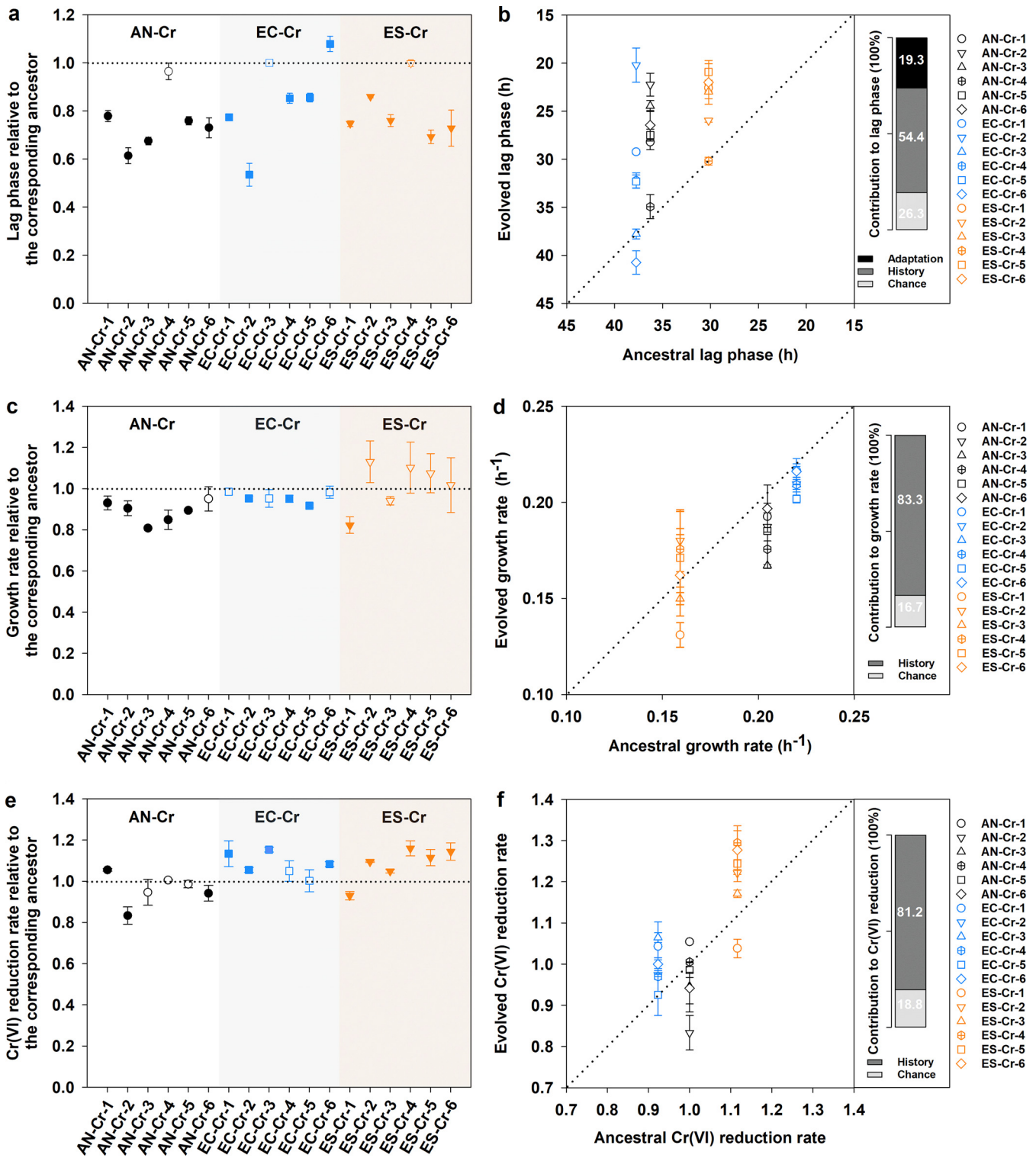
FIG 3 Cr(VI) resistance (lag phases [a] and growth rates [c]) and Cr(VI) reduction rates [60 min after addition of Cr(VI)] (e) of the Cr(VI) stress-evolved populations and contributions of the pre-existing genetic divergence, chance, and adaptation to the evolved mean lag phases (b), mean growth rates (d), and mean Cr(VI) reduction rates (f). Filled symbols in a, c, and e indicate significantly different values from the corresponding ancestor (t test, P < 0.05). Error bars represent the standard deviations for three biological replicates.

Additionally, synonymous mutations and noncoding mutations in the intergenic regions were observed in EC-Cr and ES-Cr populations only, and large deletions (about 40 kb) affecting multiple genes were found only in the AN-Cr group.

New mutations (excluding the four large deletions in AN-Cr group) were identified
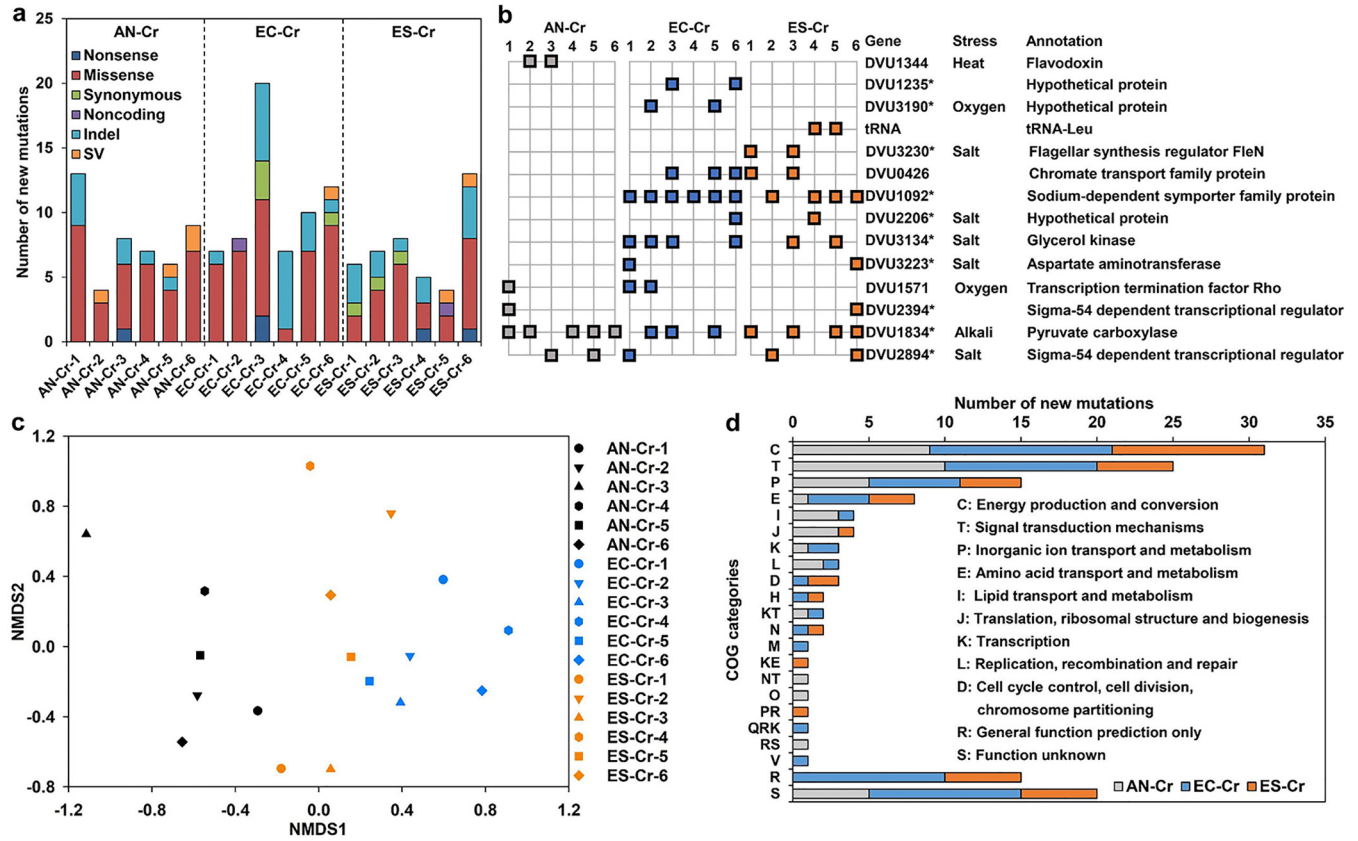
FIG 4 Analysis of the new mutations in the Cr(VI) stress-evolved populations. (a) Classification of the new mutations. SV, structural variant. (b) Detailed information of the 14 newly mutated genes found in at least two evolved populations. Genes labeled with stars were reported to have higher expression in DvH biofilms than in batch planktonic cells. (c) NMDS ordination of the combined gene level (77 genes) mutation frequencies of the new mutations (stress = 0.15). The frequencies of all the mutations in one gene were combined to represent the mutation frequency of this gene. Each symbol represents one population. (d) Categorization of the new mutations based on COG designations of the mutated genes. The full list of the COG annotations is in Table S1.

in 77 genes (Table S1). Of these, eight genes were found mutated in EC3-10 and/or ES9-11 as well (36); therefore, 69 newly mutated genes were considered unique under Cr(VI) evolution and further analyzed. Fifty-five of these newly mutated genes were mutated in only one Cr(VI) stress-evolved population, indicating the substantial variance of the mutations among the evolved populations. Fourteen of these newly mutated genes were mutated in two or more evolved populations (Fig. 4b). Among these genes, five genes were mutated in two populations within one group, including DVU1344 in the AN-Cr group, DVU1235 and DVU3190 in the EC-Cr group, and DVU_tRNA-Leu and DVU3230 in the ES-Cr group. Five genes were mutated in both EC-Cr and ES-Cr populations, including DVU0426, DVU1092, DVU2206, DVU3134, and DVU3223. DVU1571 was mutated in both the AN-Cr and EC-Cr populations, DVU2394 was mutated in both the AN-Cr and ES-Cr populations, and DVU1834 and DVU2894 were mutated in all three groups.

To examine the dissimilarities of the genomic mutation spectra in the Cr(VI)-stress evolved populations, the mutation frequencies of all new mutations at gene level (77 genes) were tested with a nonmetric multidimensional scaling (NMDS) ordination. As shown in Fig. 4c, the AN-Cr populations were distinctly separated from the EC-Cr and ES-Cr populations, while the EC-Cr and ES-Cr populations were not clearly separated. The nonparametric multivariate analysis of variance (adonis) indicated that the gene mutation spectra in the evolved populations differed substantially between AN-Cr and EC-Cr ($F = 2.86$, $P = 0.001$), AN-Cr and ES-Cr ($F = 1.55$, $P = 0.012$), and EC-Cr and ES-Cr ($F = 1.50$, $P = 0.046$) populations. These results indicated that the genetic backgrounds

of the starting clones had strong influences on the genetic evolution of DvH populations under Cr(VI) stress.

The new mutations were further analyzed based on the cluster of orthologous groups (COG) (45) to evaluate the effects of genetic backgrounds on the selection of functional targets under Cr(VI) stress (Table S1). In all three groups, the top three well-defined COG hit by mutations include energy production and conversion (C category), signal transduction (T category), and inorganic ion transport and metabolism (P category) (Fig. 4d). However, the number of mutations varied among the three groups in some COG, such as T (signal transduction) and E (amino acid transport and metabolism), and most COG hit by mutations occurred only in specific groups (Fig. 4d). Adonis analysis indicated that the COG mutated during Cr(VI) evolution differed significantly among the three groups ($F = 1.58$; $P = 0.023$). Pairwise comparisons revealed that significant difference of the mutated COG was found only between the AN-Cr group and the EC-Cr group (adonis, $F = 1.92$, $P = 0.006$). Together, these results implied the influence of prior evolution history on the selection of mutations under Cr(VI) stress at the functional level, although not as strong as that at the gene level.

Functional characterization of the chromate transporter gene DVU0426. The co-occurrence of different mutations in the same genes in multiple evolved populations implies the importance of these genes in the adaptation to Cr(VI) stress (46). DVU0426, annotated as a chromate transport family protein, was mutated in two ES-Cr (ES-Cr-1 and ES-Cr-3) and three EC-Cr (EC-Cr-3, EC-Cr-5, and EC-Cr-6) populations (Fig. 4b). According to protein structure prediction (http://smart.embl-heidelberg.de/), these mutations would likely lead to loss of one or two conserved chromate transporter domains, ChrA, in the predicted DVU0426 protein (Fig. 5a). To investigate the function of DVU0426 in Cr(VI) tolerance, two site-directed mutants, including LMD (long mutant deletion), mimicking the mutation in ES-Cr-3, and SMD (short mutant deletion), mimicking the mutations in EC-Cr-3 and EC-Cr-6, were generated. In addition, an in-frame deletion mutant (MD) lacking the entire coding sequence of DVU0426 was generated. In LMD, a small portion of the C-terminal ChrA domain of DVU0426 protein was lost, and the two ChrA domains of DVU0426 protein were completely lost in the SMD and MD constructs (Fig. 5a).

Under control conditions, the growth curves of these mutants and the parental strain (JZ001) were similar (Fig. 5b). Under the Cr(VI) stress condition, SMD and MD showed markedly (t test, $P < 0.001$) prolonged lag phases (>45.0 h) compared to the parental strain (34.0 ± 0.5 h), while the lag phase of LMD (30.8 ± 1.4 h) was significantly (t test, $P < 0.05$) shorter than that of the parental strain (Fig. 5c). All mutants had the final biomass yields comparable to that of the parental strain. The Cr(VI) reduction capabilities of these DVU0426 mutants in washed-cell experiments were not significantly different from that of the parental strain (Fig. 5d). These results demonstrated the important role of DVU0426 under Cr(VI) stress. Based on annotation, DVU0426 protein is a chromate efflux transporter, and it could extrude SO_4^{2-} due to the structural similarity between CrO_4^{2-} and SO_4^{2-} (47), the terminal electron acceptor for anaerobic respiration in DvH. We speculate that the potential loss-of-function mutations in DVU0426 could avoid sulfate starvation and therefore be beneficial under Cr(VI) stress.

DISCUSSION

Chromate is one of the most common environmental stresses due to its extensive industrial application (1, 4). In this study, experimental evolution coupled with whole-genome sequencing was employed to uncover the genetic basis of Cr(VI) adaptation in Cr(VI) stress-evolved DvH populations. By evolving three groups of genetically divergent DvH populations under the same Cr(VI) stress condition, we could dissect the contributions of adaptation, chance, and the pre-existing genetic divergence to the evolution of DvH under Cr(VI) stress.

Results from this study demonstrated that the Cr(VI) stress-evolved populations had shortened lag phases and increased Cr(VI) reduction capabilities but unchanged or
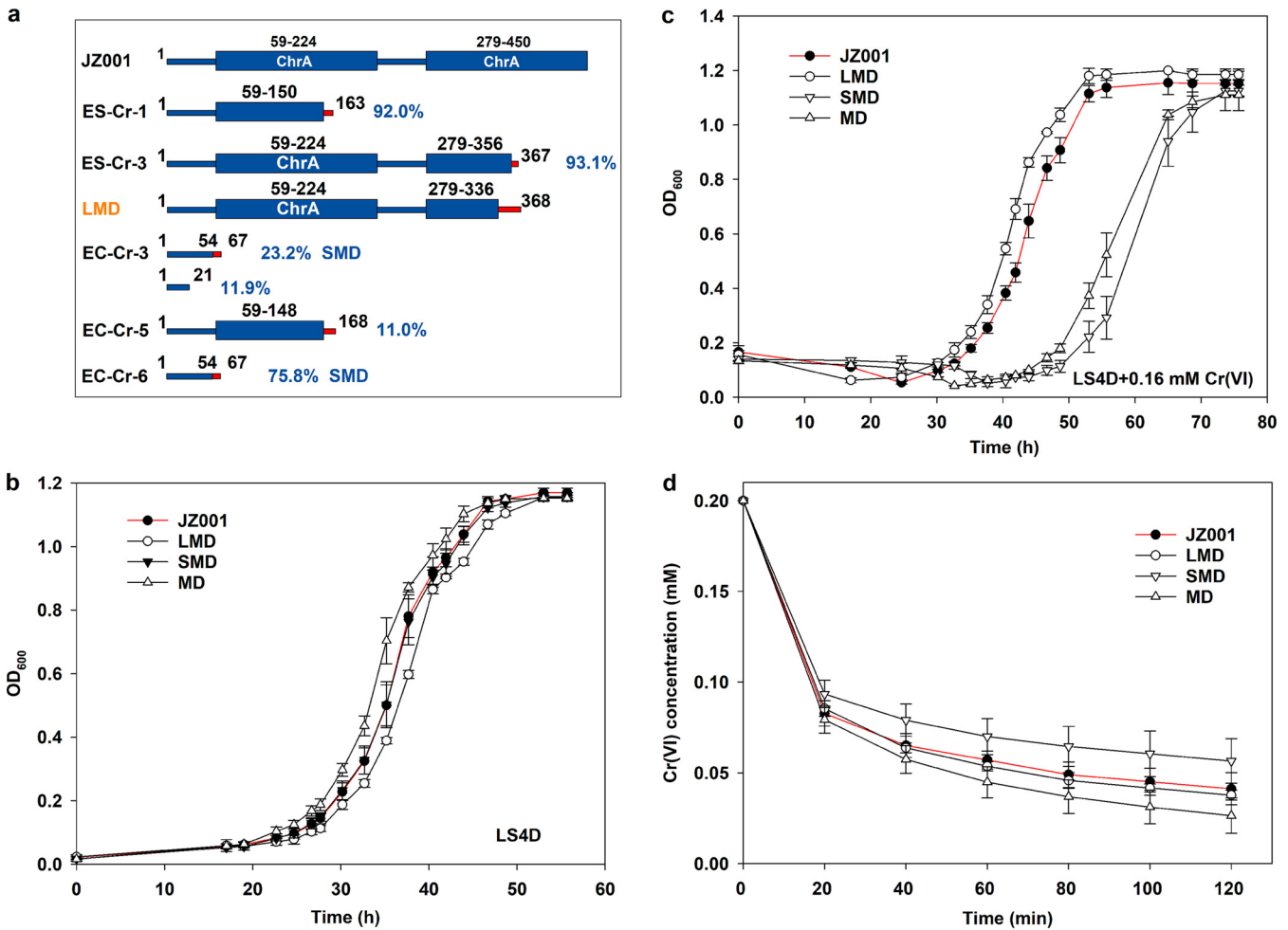
FIG 5 Cr(VI) resistance and Cr(VI) reduction ability of the DVU0426 mutants. (a) Predicted protein structures of DVU0426 in the parental strain JZ001 and evolved populations harboring DVU0426 mutations. The numbers indicate the positions of the amino acid residues. The blue boxes represent the chromate transporter domain ChrA. Red color indicates the predicted DVU0426 protein structure changes resulted from the mutations. The mutation frequencies are noted in blue. LMD, long mutant deletion; SMD, short mutant deletion. Growth curves of the parental strain JZ001 and the DVU0426 mutants in LS4D medium (b) or LS4D supplemented with 0.16 mM Cr(VI) (c) and their Cr(VI) reduction capability (d) tested with washed-cell experiments are shown. The error bars represent the standard deviations for three biological replicates.

decreased growth rates. Increased growth rates have been observed in DvH populations evolved under various stress conditions such as salt (NaCl), nitrate, and elevated temperature (36, 44, 48). In another experimental evolution experiment using the same set of starting clones as this study, all populations showed increased growth rates after evolution in elevated temperature for 1,000 generations (44). It is possible that the metabolic pathways involved in Cr(VI) stress responses are quite different from those involved in the other stress conditions. One line of evidence is that the salt stress-evolved population ES9-11 showed increased growth rates under salt stress and elevated temperature (36, 44) while showing a decreased growth rate under Cr(VI) stress in this study. Instead, one of the major traits of improved Cr(VI) tolerance is the shortened lag phase. Consistently, under Cr(VI) stress, the loss-of-function mutants of DVU0426, including MD and SMD, had prolonged lag phases; LMD, harboring the potentially functional DVU0426, showed a shortened lag phase; and all mutants had final biomass comparable to that of the parental strain. There was also a correlation between the growth phenotypes of the evolved populations under Cr(VI) stress and the DVU0426 mutations. For instance, EC-Cr-6 showed a prolonged lag phase compared to EC3-10, and it harbored a high frequency (75.8%) of a potential loss-of-function DVU0426 mutation; EC-Cr-3 grew almost the same as EC3-10, EC-Cr-5 had a shortened lag phase compared to EC3-10, and they had low frequencies

(35.1% and 11.0%, respectively) of the potential loss-of-function DVU0426 mutation; and ES-Cr-3 showed better Cr(VI) tolerance (shortened lag phase) than ES9-11, and it had an almost fixed (93.1%) DVU0426 mutation, which could increase Cr(VI) resistance, as demonstrated by the LMD mutant. ES-Cr-1 is one exception with improved Cr(VI) tolerance (shortened lag phase) compared to ES9-11 and a high frequency (92.0%) of the potential loss-of-function DVU0426 mutation. Together, these results indicated that shortened lag phase is one of the major phenotypic traits of Cr(VI) tolerance and the relationship between phenotype and genotype is complex, depending on the context of mutations within the genome.

Dissection of the contributions of the driving forces in the evolution of three traits, including lag phase, growth rate, and chromate reduction rate, demonstrated the highest contribution of the pre-existing genetic divergence in all three traits. Similarly, the pre-existing genetic divergence also had significant influences on evolution of the same set of starting clones under elevated temperature conditions (44). In addition, our study revealed the genomic changes acquired during Cr(VI) evolution and the strong influences of the pre-existing genetic divergence on the second phase of genetic evolution. A total of 14 newly mutated genes were found in more than two evolved populations (Fig. 4b), implying the importance of these genes in Cr(VI) evolution. Among these genes, DVU0426 was shown to be important for Cr(VI) tolerance (Fig. 5). Nine genes have been reported to be involved in multiple stress responses (Fig. 4b). For example, protein abundances of DVU1571 decreased and DVU3190 increased under oxidative stress (49–51); gene expression of DVU1344 increased under heat shock (52); the expression of DVU2206, DVU2894, DVU3134, DVU3223, and DVU3230 was associated with NaCl stress (36, 53, 54); and expression of DVU1834 decreased under high pH (55). Moreover, expression of 10 of these 14 genes (Fig. 4b) was higher in DvH biofilms than batch planktonic cells (56). Biofilms can protect bacteria against multiple stresses, including salt, osmolarity, pH, nutrient availability, and redox (57). Active removal and immobilization of hexavalent uranium and lead has been reported in biofilms of *D. desulfuricans* G20 (58–61). We speculate that, altogether, these 14 genes are potentially important for coping with Cr(VI) stress and might be involved in general stress responses as well.

In summary, significantly increased Cr(VI) resistance and Cr(VI) reduction were achieved in DvH populations through experimental evolution under Cr(VI) stress conditions; the pre-existing genetic divergence of the starting clones significantly influenced the phenotypic and genetic evolution of the DvH populations under Cr(VI) stress condition. Functional analysis of a newly mutated gene, DVU0426, which encodes a chromate transport family protein and was mutated in multiple ES-Cr and EC-Cr populations, demonstrated its important role in Cr(VI) tolerance. Future studies will identify the contributions of the other potentially beneficial genes in Cr(VI) tolerance. In addition, investigations about the interactions among beneficial mutations and gene regulatory networks will help to depict the molecular mechanisms of Cr(VI) tolerance and eventually promote the bioremediation strategies of Cr(VI) pollution.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Laboratory experimental evolution of *D. vulgaris* Hildenborough (DvH) under Cr(VI) stress was initiated from three starting clones. The ancestor clone (AN) was isolated from DvH originally from ATCC 29579. EC3-10 and ES9-11 were clonal isolates from populations EC3 and ES9, which had been propagated from AN under control (LS4D medium) and salt stress (LS4D plus 100 mM NaCl) conditions, respectively, for 1,200 generations (36). Six replicates from each starting clone were cultured in 10 ml defined LS4D medium (62) supplemented with 0.6 mM K_2CrO_4 at 37°C anaerobically and serially transferred every 48 h with a 1:100 dilution for 180 days to reach 600 generations. Fifty microliters of cysteine HCl solution (5% cysteine HCl [wt/vol] and 1.6 M $NaHCO_3$) was added to 10 ml LS4D medium as the reductant before the addition of K_2CrO_4 and cell inoculum. The Cr(VI) concentration of 0.6 mM was chosen to apply a moderate stress evolution condition which allows production of enough biomass for serial transfer every 48 h. The evolved populations were named AN-Cr-1 to -6, EC-Cr-1 to -6, and ES-Cr-1 to -6 (Fig. 1).

Growth phenotype analysis. Growth phenotypes of the starting clones and the Cr(VI) stress-evolved populations were determined in 10 ml control medium (LS4D) or Cr(VI) stress medium [LS4D

plus 0.16 mM Cr(VI)] with three biological replicates each. Fifty microliters of Ti(III) citrate solution [20% titanium(III) chloride (wt/vol), 0.2 M sodium citrate, and 8% sodium carbonate (wt/vol)] was used as the reductant before inoculation to minimize the abiotic reaction between Cr(VI) and the reductant (cysteine HCl) used in the evolution stage. The Cr(VI) concentration of 0.16 mM was equivalent to that used at the evolution stage (Fig. S5). Growth rate was determined as $2.303\times$ the slope of the linear portion of the growth curve obtained by plotting $\log_{10}$(optical density at 600 nm [OD_{600}]) with time (36). Lag phase was defined as the time required for the OD_{600} to reach 0.15.

Cr(VI) reduction assay with washed cells. Cr(VI) reduction capability of the starting clones and the Cr(VI) stress-evolved populations compared to the corresponding ancestor was evaluated with washed-cell experiments as previously described (63). Briefly, mid-exponential-phase cells (OD_{600} ~ 0.4) cultured in 50 ml LS4D medium were harvested by centrifugation. Ti(III) citrate solution was used as the reductant in this assay. After two rounds of washing with anoxic bicarbonate buffer (30 mM $NaHCO_3$, 62 mM sodium lactate, and 20 mM Na_2SO_4), the cells were resuspended in 0.5 ml of anoxic bicarbonate buffer. Then, the resuspended cells were added to 9.8 ml of anoxic bicarbonate buffer until the OD_{600} reached 0.4. The cell cultures were incubated at 37°C after the addition of 100 μl 20 mM K_2CrO_4. Samples (200 μl) were taken at 20-min intervals and centrifuged at 4°C for 1 min at 13,000 rpm. Then, 100 μl of the supernatants was taken for Cr(VI) quantification. Cr(VI) concentrations were determined colorimetrically (OD_{540}) by the diphenylcarbazide method with a Hach ChromaVer 3 chromium reagent powder pillow (Hach Company, Loveland, CO, USA) (64). At each time point, the Cr(VI) reduction rate of AN was set as 1, and the Cr(VI) reduction rates of EC3-10 and ES9-11 were the ratios of the amount of the Cr(VI) reduced by EC3-10/ES9-11 to that reduced by AN. In the same way, the Cr(VI) reduction rates of the Cr(VI) stress-evolved populations were transformed to the ratios of the amount of the Cr(VI) reduced by the evolved populations relative to that of the corresponding ancestor at each time point.

Whole-population sequencing. Genomic DNA was isolated from Cr(VI) stress-evolved populations with Sigma GeneElute bacterial genomic DNA kits and purified with Zymo Research genomic DNA Clean & Concentrator. The purified genomic DNAs were then fragmented to about 300 bp with a Covaris (Woburn, MA, USA) M220 focused ultrasonicator, and sequencing libraries were constructed with the Kapa HyperPrep kit. DNA fragments ranging from 320 to 480 bp were selected with Pippin (Sage Science, Beverly, MA, USA) and sequenced with a HiSeq 3000 system (Illumina, San Diego, CA, USA). The forward and reverse reads for each population were paired, merged, and then aligned to the DvH reference genome (NC_002937.3 [chromosome] and NC_005863.1 [plasmid]) in Geneious (v.9.1.8). Mutation calls (single nucleotide variants [SNVs], insertions, or deletions) were performed with Geneious, and mutations with frequencies above 10% were selected for further analysis. The mutations were classified following the previously described guidelines (65). Briefly, single-nucleotide changes in coding regions (including tRNA and rRNA genes) were classified as nonsense, missense, or synonymous if they resulted in an early stop codon, an amino acid change, or no amino acid change, respectively. Single-nucleotide mutations in the intergenic regions were classified as noncoding mutations. Insertions or deletions less than 100 bp were classified as indels. The SV class includes large structural variants (>100 bp).

Generation and characterization of site-directed mutants and an in-frame deletion mutant. DvH strain JZ001 (Δupp), which contains an in-frame deletion of the *upp* gene, was generated following a previously published method (66). The *upp* gene encodes the pyrimidine salvage enzyme uracil phosphoribosyl transferase. Wild-type DvH is sensitive to the pyrimidine analog 5-fluorouracil (5-FU) and its growth is inhibited by 5-FU. Deletion of *upp* makes the mutant resistant to 5-FU and the reintroduction of *upp* gene restores the 5-FU sensitivity in DvH, which allows the selection of a markerless mutant in DvH. With JZ001 as the parent strain, two site-directed mutants and one in-frame deletion mutant of DVU0426 were generated as previously described (37). Primers used for mutagenesis are listed in Table S2. The mutants were selected as 5-FU^r and further confirmed by sequencing of the PCR fragments harboring the mutations. Growth phenotypes and the Cr(VI) reduction abilities of the mutants were analyzed under control and Cr(VI) stress conditions as described above.

Statistical analyses. The nonmetric multidimensional scaling (NMDS) ordination (67) and the nonparametric multivariate analysis of variance (adonis) (68) were performed based on Euclidean dissimilarity using the function metaMDS and adonis in the R (v.3.6.0) package vegan (69). The contributions of adaptation, chance, and pre-existing genetic divergence to the measured traits of the Cr(VI) stress-evolved populations under Cr(VI) stress were calculated following a published method (70). Briefly, the effects of adaptation were defined as changes in the grand mean value, and 95% confidence limits were calculated by using the t distribution. Two-level nested analysis of variance (ANOVA) (three groups and six populations within each group, with three replicates of measurement per population) was conducted in R software to calculate the contribution of pre-existing genetic divergence and chance. The model was set as 'trait value ~ group/population' when using the function aov in the R package stats (71). The homogeneity of variances was checked with the Bartlett test (72). The square root of the variance component for pre-existing genetic divergence and chance was calculated in order to use units that were comparable to the mean change due to adaptation. Approximate 95% asymmetrical confidence limits were calculated for the variance components.

Data availability. The whole-genome population sequencing data were deposited in the NCBI SRA database under BioProject identifier PRJNA682882.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, JPG file, 0.5 MB.

FIG S2, JPG file, 1.1 MB.

FIG S3, JPG file, 1 MB.

FIG S4, JPG file, 1 MB.

FIG S5, JPG file, 0.9 MB.

TABLE S1, XLSX file, 0.03 MB.

TABLE S2, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

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