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Microbial Cell Factories *à la Carte*: Elimination of Global Regulators Cra and ArcA Generates Metabolic Backgrounds Suitable for the Synthesis of Bioproducts in *Escherichia coli*

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ABSTRACT Manipulation of global regulators is one of the strategies used for the construction of bacterial strains suitable for the synthesis of bioproducts. However, the pleiotropic effects of these regulators can vary under different conditions and are often strain dependent. This study analyzed the effects of ArcA, CreC, Cra, and Rob using single deletion mutants of the well-characterized and completely sequenced *Escherichia coli* strain BW25113. Comparison of the effects of each regulator on the synthesis of major extracellular metabolites, tolerance to several compounds, and synthesis of native and nonnative bioproducts under different growth conditions allowed the discrimination of the particular phenotypes that can be attributed to the individual mutants and singled out Cra and ArcA as the regulators with the most important effects on bacterial metabolism. These data were used to identify the most suitable backgrounds for the synthesis of the reduced bioproducts succinate and 1,3-propanediol (1,3-PDO). The Δ*cra* mutant was further modified to enhance succinate synthesis by the addition of enzymes that increase NADH and CO_2 availability, achieving an 80% increase compared to the parental strain. Production of 1,3-PDO in the Δ*arcA* mutant was optimized by overexpression of PhaP, which increased more than twice the amount of the diol compared to the wild type in a semidefined medium using glycerol, resulting in 24 g · liter^{-1} of 1,3-PDO after 48 h, with a volumetric productivity of 0.5 g · liter^{-1} h^{-1}.

IMPORTANCE Although the effects of many global regulators, especially ArcA and Cra, have been studied in *Escherichia coli*, the metabolic changes caused by the absence of global regulators have been observed to differ between strains. This scenario complicates the identification of the individual effects of the regulators, which is essential for the design of metabolic engineering strategies. The genome of *Escherichia coli* BW25113 has been completely sequenced and does not contain additional mutations that could mask or interfere with the effects of the global regulator mutations. The uniform genetic background of the Keio collection mutants enabled the characterization of the physiological consequences of altered carbon and redox fluxes caused by each global regulator deletion, eliminating possible strain-dependent results. As a proof of concept, Δ*cra* and Δ*arcA* mutants were subjected to further manipulations to obtain large amounts of succinate and 1,3-PDO, demonstrating that the metabolic backgrounds of the mutants were suitable for the synthesis of bioproducts.

KEYWORDS global regulators, ArcA, Cra, PhaP, 1,3-propanediol, *Escherichia coli*

Most bacteria are able to live in and adapt to many different conditions, displaying various degrees of metabolic flexibility. For example, facultative aerobes such as *Escherichia coli* can modify their metabolic pathways to optimize the use of different electron acceptors or carbon sources (1, 2). Gene expression is regulated by a network

Received 31 May 2018 **Accepted** 16 July 2018

Accepted manuscript posted online 20 July 2018

Citation Egoburo DE, Diaz Peña R, Alvarez DS, Godoy MS, Mezzina MP, Pettinari MJ. 2018. Microbial cell factories *à la carte*: elimination of global regulators Cra and ArcA generates metabolic backgrounds suitable for the synthesis of bioproducts in *Escherichia coli*. Appl Environ Microbiol 84:e01337-18. https://doi.org/10.1128/AEM.01337-18.

Editor Haruyuki Atomi, Kyoto University

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of regulators that orchestrates the transcription of genes in response to environmental stimuli, allowing cells to survive and grow in a variety of conditions. Recent studies that analyzed promoter activity across 26 different environmental conditions showed that, despite the existence of a dense and complex regulation network, a few global regulators are responsible for 70% of the changes, particularly those that affect central carbon metabolism (3). The extensive regulation network controlled by these regulators allows them to maintain cellular homeostasis by simultaneously controlling carbon flux and reducing power (4). Therefore, global regulators have been targeted to manipulate carbon metabolism and redox balance in order to generate genetic backgrounds suitable for the enhanced production of biotechnologically relevant compounds (5).

Metabolic adaptation to carbon source availability is crucial for bacteria. In *E. coli*, two main global mechanisms regulate carbon catabolism: Crp (for cyclic AMP receptor protein) (6) and Cra (for catabolite repressor/activator) (7). Cra controls carbon flux by direct repression of glycolytic enzymes and activation of tricarboxylic acid (TCA) cycle and gluconeogenic enzymes (8). In addition, *E. coli* has recently been proposed to be capable of sensing the glycolytic flux and accomplish flux-dependent regulation through the interaction of Cra with its effector 1,6-fructose bisphosphate (3, 9). The number of genes known to be part of the *cra* regulon has increased to include genes involved in stress response and nitrogen metabolism, previously unidentified transcription factors related to carbon metabolism, and even the global regulator Crp (10, 11).

The two-component system ArcAB is one of the major regulators of respiration pathways in *E. coli* in response to redox conditions (12). ArcB, a transmembrane sensor kinase, senses oxygen availability, and ArcA controls gene expression according to ArcB signaling (13). The main targets of this regulator are genes involved in central carbon metabolism. It represses TCA cycle enzymes in both microaerobic (14) and aerobic (15) conditions and activates fermentative enzymes (16). Previous studies in our laboratory have demonstrated that the inactivation of this global regulator led to an increase in the synthesis of reduced chemicals such as poly(3-hydroxybutyrate) (PHB) and ethanol (EtOH) (4, 17).

Another important two-component system, CreBC (for carbon source responsive), is a global regulator of intermediate metabolism which is active during microaerobic conditions when glycolytic carbon sources are used in minimal medium (18). Numerous gene targets have been detected for this regulator by microarray analysis (19). Deletion of *creC* has been observed to affect redox potential and central carbon metabolism in *E. coli*, creating a metabolic background suitable for succinate synthesis (20).

Metabolic flux experiments revealed a shared control of ArcAB and CreBC in the regulation of redox state and carbon substrate utilization under restricted oxygen conditions. Both systems modulate the activity of the pyruvate branch, the pentose phosphate pathway, and the TCA cycle (21). Previous studies in our group using Δ*arcA* mutants with a *creC* constitutive allele resulted in a remarkable increase in the synthesis of reduced chemicals in *E. coli* K1060 (4). The *cre* operon and *arcA* are adjacent in chromosomal location in a genomic region that also contains the gene coding for a third transcription factor, Rob, located next to the *cre* operon. Rob activates several genes belonging to the overlapping MarA/SoxS/Rob regulon, implicated in antibiotic and heavy metal resistance, organic solvent tolerance, and carbon metabolism, and has been shown to affect TCA cycle and glycolysis enzymes (22). Although the effects of Rob have not been thoroughly investigated, it reportedly activates regulatory proteins, suggesting that its regulatory effects could be more extensive (23). The known characteristics of this little-studied regulator make Rob a potentially interesting target for metabolic manipulations.

Other global regulators have also been observed to affect the tolerance of different strains of *E. coli* to diverse stress factors. Cra reportedly affects stress-related genes (10). The absence of ArcA caused an increase in sensitivity to organic solvents, and in contrast, deletion of *crp* in the same strain caused higher tolerance to *n*-hexane (24). ArcA has also been demonstrated to control the *gadE-mdtEF* operon (25) involved in acid and multidrug resistance (26). These results suggest that manipulation of global

regulators can affect the tolerance of bacteria to different chemicals, including compounds of biotechnological interest.

Although several studies have analyzed the effect of global regulator manipulations on metabolism and the synthesis of several bioproducts, many of them have utilized *E. coli* strains with unsequenced genomes (4, 20) or strains carrying mutations in genes involved in central metabolism (27). These factors hinder the correct interpretation of the effects of global regulator manipulations *per se*, since the pleiotropic and complex effects of these regulators over metabolism and physiology can vary in different genetic backgrounds. Based on studies performed in *E. coli* MC4100, it was believed that ArcA was exclusively involved in the maintenance of the redox state in microaerobiosis, since no significant changes in flux analysis were seen under fully aerobic and anaerobic conditions (28). However, metabolic flux analysis of chemostat cultures of strain BW25113, using glucose as a carbon source, revealed important changes in the TCA cycle, in the pentose phosphate pathway, and also in the pyruvate dehydrogenase complex in *arcA* and/or *arcB* mutants grown in both microaerobic (29) and aerobic (15) conditions. Similar differences have been observed when comparing the effects of other global regulators, such as *cre*, in *E. coli* strains in several growth conditions (20). A comparative study of the transcriptional and metabolic effects of the Δ*cra* mutation in two *E. coli* strains showed that the Δ*cra* deletion led to very different phenotypes in strains JM109 and BL21 due to differences in their regulatory targets, such as the *bet* operon genes, that were affected by the Δ*cra* deletion in JM109 but not in BL21 (30). This strain dependence complicates the identification of the changes that can be attributed to each global regulator, so comparative studies in a uniform genetic background are needed for a better understanding of the effects of the manipulation of the global regulators on cell physiology and particularly on central metabolism.

In the present study, we investigated the effect caused by the absence of global regulators ArcA, CreC, Rob, and Cra using single-knockout mutants of *E. coli* BW25113 from the Keio collection. This strain has a well-defined pedigree not subjected to mutagens, and its genome has been completely sequenced (31, 32). The effects of the absence of each regulator on physiology and growth were analyzed at different oxygen concentrations by metabolic profiling and stress tolerance assays. The suitability of the different metabolic backgrounds for the synthesis of several bioproducts was tested, and the strains with the best performance were subjected to further manipulation to enhance the production of succinate and 1,3-propanediol (1,3-PDO). The results obtained contribute to a better understanding of the effects of the most relevant global regulators involved in central carbon metabolism and redox regulation in *E. coli* and reveal suitable genetic backgrounds for the production of added-value compounds.

RESULTS AND DISCUSSION

Effects of the absence of global regulators on metabolism and physiology of *E. coli* BW25113. (i) Metabolic profile of global regulator mutants. When the synthesis of organic acids was studied in cultures of each mutant grown in M9 medium supplemented with 5 g liter^{-1} glucose under low-aeration conditions, all global regulators were observed to affect metabolite distribution. The Δ*rob* strain showed an increase in lactic, formic, and acetic acids, while increases in the synthesis of acetate and lactate were also detected in the Δ*creC* and Δ*arcA* mutants, respectively, compared to the control strain (Fig. 1A). Elimination of Cra seemed to affect the formation of all acids. Significant increases were seen for pyruvate (1.6-fold), succinate (2.5-fold), and lactate (1.5-fold) compared to the wild type (Fig. 1A). Levels of glucose consumption were similar for all strains (see Fig. S1 in the supplemental material), but biomass formation was lower for the Δ*arcA* and Δ*cra* mutants compared to the wild-type strain (Fig. 1A). These data suggest that carbon distribution was directed toward the synthesis of organic acids in Δ*cra* mutants and mostly to lactate in the Δ*arcA* background. This could be explained by an increase in carbon flux through glycolysis and repression of the TCA cycle that has been previously reported in Δ*cra*

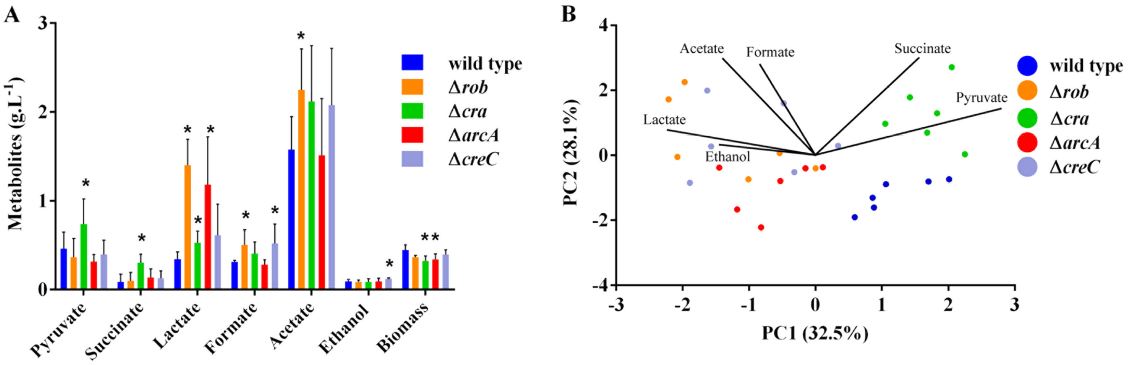

FIG 1 Metabolic profiles of global regulator mutants. Cultures were grown in M9 supplemented with glucose at 5 g liter^{-1} in shaken flasks under low-aeration conditions. The strains used were *E. coli* BW25113 as the control (wild type) and its mutant derivatives (Δ*rob*, Δ*cra*, Δ*arcA*, and Δ*creC*). (A) Results represent the mean values ± the standard deviations of each metabolite and biomass measured in the supernatants after 24 h from at least three independent replicates. An asterisk (*) indicates significant differences when comparing mutant strains to the wild-type strain (*t* test, $P < 0.05$, $n = 6$). (B) Unsupervised PCA was applied to the organic acid profile of all strains using metabolite concentrations relativized to the final biomass (g metabolite · g CDW^{-1}). The original variables are displayed in the graphic, and values of triplicates from at least two independent experiments were used ($n = 6$).

mutants (7) and by the funneling of carbon toward the synthesis of reduced compounds in the case of the Δ*arcA* mutant (4).

When EtOH was measured under low-aeration conditions, no changes in alcohol production were detected in the mutants except for the Δ*creC* strain, in which a slight but significant increase was observed compared to the control strain (0.12 ± 0.01 g liter^{-1} and 0.09 ± 0.02 g liter^{-1}, respectively) (Fig. 1A). In addition, the metabolic profiles of nonaerated cultures of the Δ*cra* and Δ*rob* mutants were also determined, since the information available on the effects of these regulators under this condition is scarce for Cra and is not available for Rob. Although the Δ*rob* strain showed important increases in the synthesis of lactate, formate, and acetate, accompanied by a modest decrease in biomass when grown under low aeration (Fig. 1A), under nonaerated conditions the Δ*rob* mutant did not show significant differences compared to the control strain (Table 1). In contrast, the increase in acid formation observed for the Δ*cra* mutant compared to the control was even more marked in nonaerated cultures than under low aeration. Fermentation acids increased around 2-fold, while a 30% reduction in biomass formation was observed for the nonaerated cultures of the Δ*cra* strain compared to the wild type (Table 1). This is similar to the results observed under low-aeration conditions (Fig. 1A) and suggests that carbon is being used for acid synthesis rather than for biomass production in this mutant under both conditions tested.

Comparison with the results reported in previous studies showed that, whereas the results obtained using the same strains were consistent, such as the higher acid production in the Δ*cra* mutants (33), the results obtained with other strains varied. For example, the increase in the formation of organic acids in the Δ*arcA* and Δ*creC* mutants reported here was also observed in K1060 derivatives containing the same mutations (20, 34), but contrasting results were found when EtOH synthesis data were compared. While the Δ*creC* derivative of BW25113 displayed higher EtOH amounts than the wild

TABLE 1 Organic acid production by wild-type, Δ*cra*, and Δ*rob* strains under nonaerated conditions[a]

Strain	Metabolite synthesis (g · liter^{-1})						Glucose consumption (g · liter^{-1})
	Pyruvate	Succinate	Lactate	Formate	Acetate	Biomass	
Wild type	0.03 ± 0.01	0.44 ± 0.08	0.97 ± 0.14	1.2 ± 0.2	0.8 ± 0.1	0.26 ± 0.02	4.4 ± 0.6
Δ*cra*	0.06 ± 0.01*	0.94 ± 0.14*	1.70 ± 0.50*	1.7 ± 0.3*	1.3 ± 0.4*	0.19 ± 0.01*	5.3 ± 0.3
Δ*rob*	0.03 ± 0.01	0.47 ± 0.70	1.00 ± 0.08	1.1 ± 0.4	0.9 ± 0.2	0.22 ± 0.03	4.7 ± 0.3

[a]Cultures were grown in M9 medium supplemented with glucose at a 5 g · liter^{-} final concentration under nonaerated conditions. The strains used were *E. coli* BW25113 (wild type) and its Δ*cra* and Δ*rob* derivatives. Results represent means ± the standard deviations from independent replicates ($n = 6$). *, significant differences when comparing mutant strains and the wild-type strain (*t* test, $P < 0.05$).

TABLE 2 Tolerance assays for global regulator mutant strains under different oxygen availability conditions[a]

Strain	Tolerance (%)								
	FA				LA				Plates
	EtOH	1,3-PDO	ButOH	NaCl	EtOH	1,3-PDO	ButOH	NaCl	(TB)
Wild type	28.0 ± 0.3	48 ± 15	2 ± 1	71 ± 10	7 ± 2	28 ± 9	35 ± 4	43 ± 8	87 ± 15
Δ*rob*	26 ± 5	37 ± 1	13 ± 6	70 ± 7	7 ± 2	32 ± 7	41 ± 2	37 ± 5	86 ± 4
Δ*cra*	NG	36 ± 1	NG	50 ± 8	NG	NG	NG	50 ± 5	45 ± 16*
Δ*creC*	25 ± 13	36 ± 4	2 ± 2	75 ± 14	8 ± 2	6 ± 3*	37 ± 2	54 ± 9	78 ± 15
Δ*arcA*	37 ± 11	39 ± 4	3 ± 1	42 ± 10*	9 ± 2	42 ± 5*	39 ± 5	44 ± 9	26 ± 9*

[a]All values are expressed as percentages of bacterial growth relative to growth in medium with no stress agent. For solvent and salt stress, shaken-flask cultures were grown in M9 supplemented with glucose at 5 g · liter^{-1} under both low-aeration and fully aerated conditions. The strains assayed were *E. coli* BW25113 (wild type) and its Δ*cra* and Δ*arcA* derivatives. Cells were exposed to different stress agents for 24 h at 37°C. Redox stress, induced by toluidine blue, is expressed as a percentage of the colony diameter formed in M9 agar medium with glucose at 5 g · liter^{-1} plus toluidine blue at 0.02% relative to growth in the same medium with no stain. Results represent means ± the standard deviations from 3 independent measurements (10 in the case of TB). *, significant differences when comparing mutant strains and the wild-type strain (*t* test, $P < 0.05$). FA, full aeration; LA, low aeration. The concentrations of stress agents used, expressed in % (wt/vol), were as follows: EtOH (ethanol), 4.8% in FA and 4.4% in LA; 1,3-PDO (1,3-propanediol), 7% in FA and LA; ButOH (butanol), 0.8% in FA and 0.6% in LA; NaCl, 3.5% in FA and LA; and TB (toluidine blue), 0.02%. NG, no growth.

type, the EtOH levels produced by the Δ*creC* derivative of strain K1060 and its parent strain did not differ (20). The opposite was observed when the Δ*arcA* mutants were analyzed, since while the K1060 derivative had increased EtOH production (35), no differences were observed for the Keio collection strain in the present study.

Unsupervised principal component analysis (PCA) was applied to the metabolic profiles obtained in low-aeration glucose cultures. Two principal components interpreted 60% of the variance. The analysis showed that all regulators affected carbon distribution in *E. coli* BW25113 under the conditions tested, since a clear separation was found between all mutations and the wild-type strain. In addition, the Δ*cra* mutant displayed an evident separation not only from the wild type but also from the Δ*arcA,* Δ*creC*, and Δ*rob* strains (Fig. 1B).

(ii) Tolerance assays. Previous studies performed with strains containing variants of different global regulators showed that these changes can affect the tolerance to several chemicals and other stressors (10, 24). Industrial bioprocesses are associated with high-density cultures in which cells are exposed to several stresses, including osmotic and redox stress and high concentrations of the bioproducts, some of which can be cytotoxic (36). The possibility that the regulators analyzed in in the present study could affect tolerance to different stressors was studied in order to assess the potential fitness of the mutants in biotechnological processes. The tolerance of the mutants to osmotic stress (NaCl) and to the bioproducts EtOH, butanol (ButOH), and 1,3-PDO was determined. Sensitivity to redox stress caused by toluidine blue was also determined, since the Dye phenotype, an increase in sensitivity to this redox dye attributed to reactive oxygen species (ROS) accumulation due to a higher rate of oxygen consumption (37), was one of the first effects attributed to the loss of ArcA (37).

Only small differences in tolerance were seen for the different mutants under all conditions tested, except for the Δ*cra* strain, which showed an increased sensitivity to EtOH and ButOH and to toluidine blue. The tolerance to osmotic stress of this strain was comparable to the wild type under full-aeration conditions and slightly but significantly lower under full-aeration conditions (Table 2). In contrast, the Δ*arcA* deletion had no relevant negative effects on tolerance, except for the well-known increase in sensitivity to toluidine blue (37) and a little decrease in growth in the presence of 3.5% (wt/vol) NaCl in restricted aeration (Table 2). Moreover, a slightly increased tolerance to 7% (wt/vol) 1,3-PDO was detected for this strain (Table 2). In the case of the Δ*creC* strain, the only discernible effect was a slight increase in the sensitivity to 1,3-PDO under low aeration (Table 2). Finally, Δ*rob* mutants did not exhibit appreciable differences compared to the wild type.

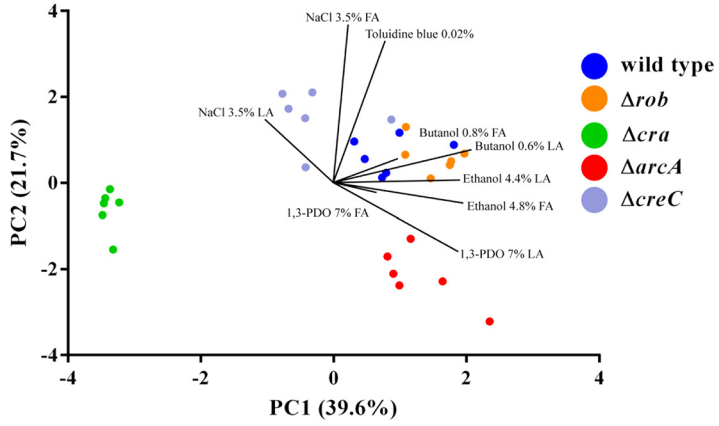

FIG 2 Tolerance assays of global regulator mutants. Unsupervised PCA was applied to the response of all strains to different stress agents. The original variables are displayed in the graphic, and values of triplicates from at least two independent experiments were used ($n = 6$). FA, full aeration; LA, low aeration. The concentrations of stress agents are expressed as the percentage (wt/vol).

The PCA, including all tolerance results, showed that the Δ*cra* mutant, which was extremely sensitive to many of the compounds tested, especially under low-aeration conditions, was clearly separated from the rest by the maximum distance, followed by the Δ*arcA* strain, while the distribution of the Δ*rob* and Δ*creC* strains partially overlapped with wild-type *E. coli*. PC1 and PC2 explained 61% of the total variance in this analysis (Fig. 2). The increased sensitivity of the Δ*cra* knockout strain could be explained by changes in the stress response, since several stress response regulators have been identified as putative Cra targets (10). In addition, a decreased expression of the *bet* operon, responsible for osmotic homeostasis, has been reported in JM109 Δ*cra* mutants (30). The increased sensitivity to toluidine blue observed for Δ*cra* mutants in this study could be an indicator of redox stress and increased ROS production, similar to the Dye phenotype observed in Δ*arcA* strains (37). The unexpected lack of sensitivity to the different compounds observed for the Δ*rob* mutant could be due to the characteristics of the MarA/SoxS/Rob regulon. These regulators share the control of many stress-related genes (22), so it is possible that the absence of Rob can be compensated by the activity of MarA or SoxS.

Identification of suitable genetic backgrounds for bioproduct synthesis. Following the analysis of the metabolite profiles and tolerance of the mutants, the effect of each deletion was studied in several strains containing heterologous genes that increase the synthesis of EtOH or allow the production of the nonnative products PHB and 1,3-PDO. The production of these compounds was assayed in cultures of each mutant grown for 24 h in different media and aeration conditions at 37°C.

(i) Ethanol. EtOH production was measured in all mutants containing a plasmid that carries *adhE* from *Leuconostoc mesenteroides* in cultures grown under low-aeration conditions in lysogeny broth (LB) supplemented with glucose or glycerol at 10 g · liter^{-1} final concentration. When glucose was used, Δ*arcA* and Δ*creC* mutants produced 2-fold more EtOH and a higher yield compared to the wild type (Table 3). No differences were observed for the Δ*rob* and Δ*cra* mutants compared to the wild-type strain. The improved EtOH synthesis in Δ*arcA* glucose cultures in this work was similar to the increase observed in other Δ*arcA* strains (34) and has been related to a higher availability of reducing power. The increase in EtOH synthesis observed for the Δ*creC* mutant was not reported in other strains but is consistent with the effect of CreC observed in the nonrecombinant strains reported here (Fig. 1A).

In cultures supplemented with glycerol, the wild-type, Δ*rob*, and Δ*creC* strains had higher EtOH synthesis than the same strains grown using glucose. The larger amounts of EtOH produced with glycerol compared to glucose cultures have been often observed and are attributed to the fact that glycerol is a more reduced carbon source

TABLE 3 Ethanol production in strains expressing *adhE*[a]

Strain	Concn (g · liter^{-1})		Specific production (mol EtOH · mol CDW^{-1})		Yield (mol EtOH · mol S^{-1})		Substrate consumption (g · liter^{-1})	
	Glucose	Glycerol	Glucose	Glycerol	Glucose	Glycerol	Glucose	Glycerol
Wild type	0.30 ± 0.04	0.73 ± 0.06	0.29 ± 0.03	0.56 ± 0.03	0.3 ± 0.1	0.45 ± 0.14	5 ± 1	3.5 ± 1.0
Δ*rob*	0.32 ± 0.07	0.85 ± 0.12	0.26 ± 0.06	0.65 ± 0.06	0.2 ± 0.1	0.42 ± 0.03	6 ± 1	4.1 ± 0.8
Δ*cra*	0.39 ± 0.02	0.47 ± 0.02*	0.34 ± 0.03	0.42 ± 0.04*	0.4 ± 0.2	0.27 ± 0.02*	5 ± 1	3.5 ± 0.4
Δ*arcA*	0.67 ± 0.12*	0.38 ± 0.01*	0.53 ± 0.11*	0.28 ± 0.05*	0.95 ± 0.20*	0.30 ± 0.03*	4 ± 1	2.6 ± 0.3
Δ*creC*	0.58 ± 0.09*	0.72 ± 0.07	0.57 ± 0.06*	0.55 ± 0.05	0.5 ± 0.1*	0.39 ± 0.08	5 ± 1	3.8 ± 1.0

[a]Cultures were grown in shaken flasks using LB medium supplemented with glycerol or glucose (both at a 10 g · liter^{-1} final concentration) under low-aeration conditions for 24 h. Results represent means ± the standard deviations from at least three independent replicates. All strains harbored the pET_{Lm} plasmid (carrying *adhE* from *L. mesenteroides*). Expression of *adhE* was induced by the addition of IPTG at 0.5 mM when cultures reached the early exponential phase (OD_{600} = 0.8). *, significant differences when comparing mutant strains and the wild-type strain (*t* test, $P < 0.05$). CDW, cell dry weight; S, substrate.

than glucose (38). Unexpectedly, the Δ*arcA* glycerol cultures produced less EtOH than the corresponding glucose cultures, and both Δ*arcA* and Δ*cra* strains produced even less EtOH than the wild type when we compared the strains grown on glycerol (Table 3). These results are in contrast to those obtained with a Δ*arcA* derivative of K1060 (4) and can be related to the low glycerol consumption displayed by the Δ*arcA* mutant used in the present study (Table 3).

(ii) Poly(3-hydroxybutyrate). The effect of the different mutations on the synthesis of the biodegradable thermoplastic PHB was also tested. Since *E. coli* does not produce this polymer naturally, a plasmid containing the PHB synthesis genes from *Ralstonia eutropha* (also named *Cupriavidus necator*) was introduced in the different global regulator mutants and in the control strain. The synthesis of this reserve polymer produced by many bacterial species involves the use of carbon and reducing power (17), so mutations that affect carbon fluxes and the redox state of the cells were expected to also affect PHB synthesis. However, cultures of all global regulator mutant derivatives of BW25113 accumulated amounts of PHB similar to the wild type when grown in LB supplemented with glucose under low aeration. Previous work performed in K1060 showed an increase in the polymer content of the Δ*arcA* mutants (17), but no differences in PHB cell content were observed when the Keio collection Δ*arcA* mutant was grown under similar conditions (see Fig. S2 in the supplemental material). The discrepancies in the results obtained with the *E. coli* BW25113 and K1060 Δ*arcA* derivatives can be attributed to strain differences and point once again to the strain dependence of the global regulator knockout mutant phenotypes.

(iii) 1,3-PDO. 1,3-Propanediol (1,3-PDO) is a high-value bulk chemical with many industrial applications, especially the production of polytrimethylene terephthalate (39). 1,3-PDO can be obtained by microbial fermentation of glycerol, the main by-product of biodiesel production, and thus is an inexpensive and readily available substrate (40). Genes for 1,3-PDO synthesis from *Klebsiella pneumoniae* GLC29 (41) were used to promote the synthesis of this diol from glycerol; this was studied in cultures of all global regulator mutant strains grown in M9 or LB in full and low aeration. When cultures were grown in M9, no significant differences in 1,3-PDO concentration were observed under any aeration condition. However, a slightly augmented final concentration of 1,3-PDO was detected in the Δ*arcA* mutant in low aeration despite a 44% reduction in glycerol consumption (Table 4). A similar increase in 1,3-PDO titer was observed for the Δ*cra* mutant, but a higher production per gram of biomass was obtained in this strain due to a decrease in growth, probably due to its reduced capability to use gluconeogenic carbon sources (8). Lastly, the Δ*creC* mutant showed a marked decrease in glycerol consumption compared to the parental strain in restricted and fully aerated M9 cultures (about 50 and 40%, respectively) (Table 4). This is in line with previous observations that described an impaired growth of Δ*creC* derivatives of DH5α in minimal medium supplemented with glycerol as the sole carbon source (42).

When synthesis of 1,3-PDO was monitored in LB cultures, no differences between strains were observed in low-aeration conditions (data not shown). In contrast, when

TABLE 4 Production of 1,3-PDO in strains expressing the *dha* operon[a]

Strain	1,3-PDO concn (g · liter^{-1})		Specific production (mol 1,3-PDO · mol CDW^{-1})		Yield (mol 1,3-PDO · mol Gly^{-1})		Substrate consumption (g · liter^{-1})	
	LA	FA	LA	FA	LA	FA	LA	FA
Wild type	0.72 ± 0.08	1.5 ± 0.5	1.50 ± 0.13	0.40 ± 0.07	0.63 ± 0.17	0.17 ± 0.09	1.9 ± 0.3	19 ± 1
Δ*rob*	0.73 ± 0.10	1.00 ± 0.03	1.30 ± 0.12	0.47 ± 0.08	0.53 ± 0.12	0.10 ± 0.01	1.9 ± 0.4	14 ± 3
Δ*cra*	0.88 ± 0.11	1.5 ± 0.3	2.40 ± 0.42*	0.85 ± 0.21*	0.71 ± 0.17	0.23 ± 0.02	1.6 ± 0.3	8 ± 1*
Δ*arcA*	0.90 ± 0.02	1.6 ± 0.5	1.50 ± 0.12	0.56 ± 0.08	0.81 ± 0.10	0.14 ± 0.07	1.0 ± 0.2*	15 ± 3
Δ*creC*	0.66 ± 0.07	1.2 ± 0.3	1.40 ± 0.06	0.35 ± 0.05	0.85 ± 0.03	0.11 ± 0.02	0.9 ± 0.1*	12 ± 1*

[a]Cultures were grown in shaken flasks and M9 medium supplemented with glycerol at 10 and 30 g · liter^{-1} for low-aeration and fully aerated conditions, respectively, for 24 h. Results represent means ± the standard deviations of three independent replicates. All strains harbored plasmid pBBR1MCS-2::*dha* (carrying the *dha* operon from *K. pneumoniae*). *, significant differences when comparing mutant strains and the wild-type strain (*t* test, $P < 0.05$). Gly, glycerol; LA, low aeration; FA, full aeration; CDW, cell dry weight.

fully aerated experiments were conducted, a marked increase was found in Δ*arcA* mutants, which produced >2-fold more 1,3-PDO than all other strains, reaching 10.0 ± 0.2 g liter^{-1} in 24-h batch cultures (Fig. 3). Glycerol consumption was highly increased in this mutant compared to the wild type, but the substrate was apparently used mostly for the synthesis of the diol, since both 1,3-PDO yields and specific production were increased in the Δ*arcA* mutants (Table 5). No significant differences in diol concentration were observed between the rest of the mutants and the wild type, including the Δ*cra* strain, which had evidenced higher 1,3-PDO-specific production in minimal medium (Table 4).

Considering that 1,3-PDO synthesis requires reducing power, it is not surprising that the Δ*arcA* mutants produced larger amounts of this compound. These results are in accordance with the increased synthesis of other reduced metabolites observed in K1060 Δ*arcA* mutants (43). Unexpectedly, the greatest changes took place in fully aerated conditions and not under low-oxygen conditions, as would be expected considering the results obtained with K1060. Previous metabolic characterizations of the Keio collection Δ*arcA* mutant revealed important changes in the flux through the TCA cycle under aerobic conditions (15), leading to a greater availability of reducing power that could be funneled in turn to 1,3-PDO synthesis.

Multivariate analysis. Taking into account all variables observed, the global regulator mutants displayed different phenotypes in a variety of conditions, probably due to the multiplicity of regulatory targets. The results obtained from the metabolic profiles, the tolerance assays, the production of nonnative metabolites (see Table S1 in

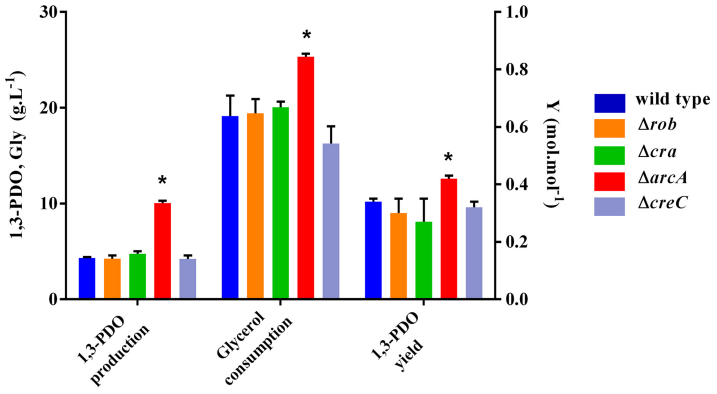

FIG 3 Production of 1,3-propanediol in recombinant *E. coli* strains. Cultures were grown in shaken flasks in LB supplemented with glycerol at 30 g liter^{-1} under fully aerated conditions for 24 h. Results represent the mean value ± the standard deviations of three independent replicates. The strains used were *E. coli* BW25113 as the control (wild type) and its mutant derivatives (Δ*rob,* Δ*cra,* Δ*arcA*, and Δ*creC*). All bacteria harbored plasmid pBBR1MCS-2::*dha* (carrying the genes for 1,3-PDO synthesis). Yield corresponds to 1,3-PDO on glycerol. An asterisk (*) indicates significant differences when comparing mutant strains and the wild-type strain (*t* test, $P < 0.05$).

TABLE 5 Effects of PhaP on the production of 1,3-PDO[a]

Strain	Yield (mol 1,3-PDO · mol Gly^{-1})	Glycerol consumption (g · liter^{-1})	Specific production (mol 1,3-PDO · mol CDW^{-1})
Wild type	0.39 ± 0.01	16.0 ± 0.4	0.62 ± 0.03
Δ*cra*	0.32 ± 0.02*	12.0 ± 0.6	0.45 ± 0.04*
Δ*arcA*	0.46 ± 0.02*	32 ± 4*	1.40 ± 0.15*
Wild type+PhaP	0.44 ± 0.01	20.0 ± 0.2	0.92 ± 0.03
Δ*arcA*+PhaP	0.73 ± 0.01*	28 ± 2*	1.80 ± 0.12*

[a]Cultures were grown in LB medium supplemented with glycerol at 30 g · liter^{-1} in shaken flasks under fully aerated conditions for 24 h. Results represent means ± the standard deviations of triplicates from at least two independent cultures. The strains used were *E. coli* BW25113 as a control and its Δ*cra* and Δ*arcA* derivatives. All strains harbored plasmids pBBR1MCS-2::*dha* (carrying the genes for 1,3-PDO synthesis) and pPhaP328 (carrying *phaP* from *Azotobacter* sp. FA8). Expression of *phaP* was induced in the wild type+PhaP and Δ*arcA*+PhaP strains by the addition of *m*-toluic acid at 0.5 mM when the cultures reached the early exponential phase (OD_{600} = 0.8). *, significant differences when comparing mutant strains and the wild-type strain (*t* test, $P < 0.05$). Gly, glycerol; CDW, cell dry weight.

the supplemental material) and the growth of all strains on minimal medium (see Fig. S3 in the supplemental material) were used to perform a descriptive multivariate analysis to search for patterns that could be associated with each mutant.

In the PCA, PC1 and PC2 explained more than 44% of the total variance and revealed well-defined groups. While during the analysis of each particular set of conditions the strains showing the greatest deviations from the control varied, when all parameters were simultaneously considered in an integral analysis the greatest distances were clearly observed between the wild type and the strains with the Δ*cra* and Δ*arcA* deletions (Fig. 4A). On the other hand, the Δ*rob* mutants only exhibited differences in organic acid formation compared to the control strain, and this was reflected in an overlap between the two strains on the PCA. Lastly, the Δ*creC* strain also overlapped with the Δ*rob* strain and the wild type (Fig. 4A), in spite of some significant differences found when comparing some of the individual characteristics of these strains, such as the production of some acids and ethanol. A hierarchical clustering analysis (HCA) of all data was able to distinguish separate groups corresponding to the wild type and each of the mutants (Fig. 4B). The distances between the clusters were congruent with the separations observed in the PCA, since the Δ*cra* mutant displayed the maximum distance from the wild type, followed by the Δ*arcA* mutant. The wild type, Δ*rob*, and Δ*creC* strains were clustered in distinct but closely related groups.

The different multivariate analyses, including PCA and HCA, showed specific patterns for each mutation despite the extensive regulation exerted by the global transcription factors and their coregulation of some gene targets (44, 45). Discriminant

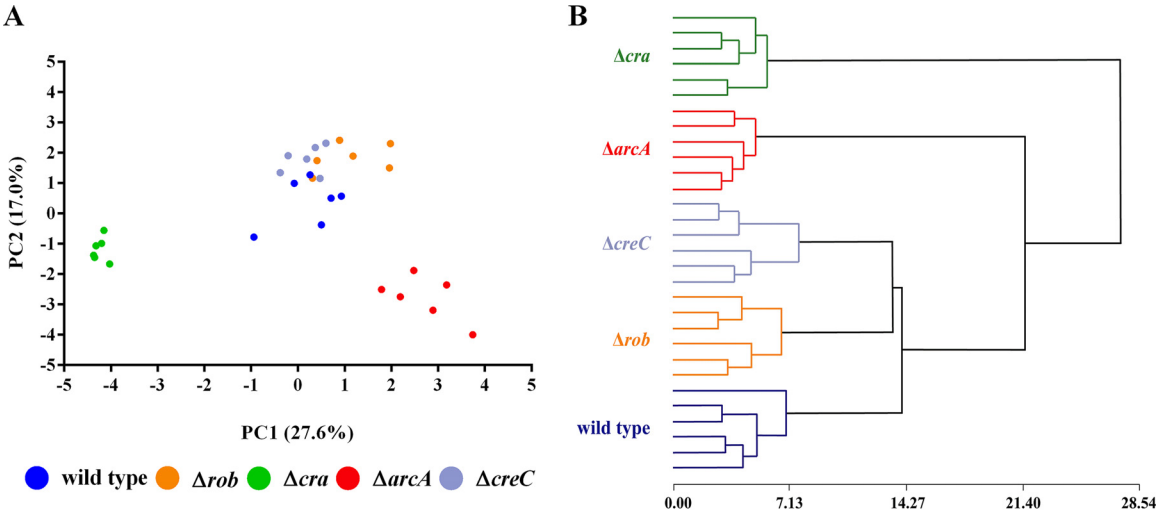

FIG 4 Multivariate analysis of the effect caused by the absence of different global regulators on *E. coli* BW25113. Descriptive multivariate analysis was performed by PCA (A) and HCA (B). Both statistics were based on 21 variables, including production of native and nonnative metabolites, growth on M9, and tolerance assays (see Table S1 and Fig. S3 in the supplemental material for detailed information). Values of triplicates from two independent experiments were used (n = 6).

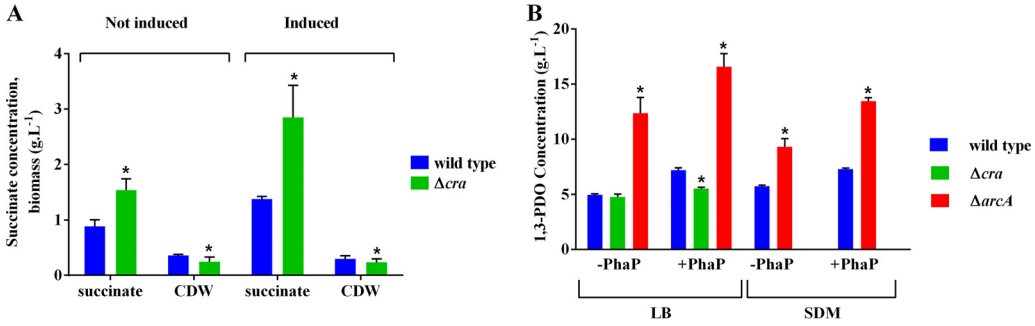

FIG 5 Production of succinate and 1,3-PDO in global regulator mutants. (A) *E. coli* BW25113 (wild type) and its Δ*cra* derivative, both carrying plasmids pSBF2 (carrying *fdh1* from *C. boidinii*) and pEcPck (carrying *pck* from *E. coli*), were grown in screw-cap tubes filled with M9 medium supplemented with glucose at 5 g liter^{-1} and yeast extract at 0.5 g liter^{-1} under nonaerated conditions for 24 h. The expression of *fdh1* and *pck* from pSBF2 and pEcPck was induced by 0.5 mM IPTG added to the medium at the onset of the cultivation. CDW, cell dry weight (g liter^{-1}). (B) *E. coli* BW25113 (wild type) and its Δ*cra* and Δ*arcA* mutant derivatives, all harboring plasmids pBBR1MCS-2::*dha* (carrying the genes for 1,3-PDO synthesis from *K. pneumoniae*) and pPhaP328 (carrying *phaP* from *Azotobacter* sp. FA8), were grown in shaken flasks using LB or SDM supplemented with glycerol at 30 g liter^{-1} under fully aerated conditions for 24 h. LB expression of PhaP was induced by the addition of 0.5 mM *m*-toluic acid when cultures reached the early exponential phase (OD_{600} = 0.8). −PhaP, cultures grown without *phaP* induction (i.e., cultures grown in the absence of *m*-toluic acid); +PhaP, cultures grown in the presence of inducer. An asterisk (*) indicates significant differences when comparing mutant strains and the wild-type strain (*t* test, $P < 0.05$).

analysis also yielded similar results (see Fig. S4 in the supplemental material). The two global regulators that showed the most distinctive effects were Cra and ArcA, an observation in agreement with studies that indicated that Cra is the main regulator of central carbon metabolism (46) and that ArcA controls either directly or indirectly a very important part of all genes in *E. coli* (47). Taking into account the physiological background created by each of the global regulator mutations, the Δ*cra* and Δ*arcA* mutants can be considered suitable for the synthesis of some bioproducts.

Metabolic manipulation of the Δ*cra* mutant for enhanced succinate production. The metabolic profiles of the Δ*cra* knockout strain showed increased succinate synthesis under both low-aeration and nonaerated conditions, but the greatest differences were observed in the nonaerated cultures. A strategy that was observed to increase succinate in other strains (20) was used to improve the production of this valuable compound in the Δ*cra* background. Briefly, *fdh1*, encoding formate dehydrogenase 1 from the yeast *Candida boidinii*, was overexpressed to convert the excess formic acid produced by the Δ*cra* mutants into CO_2 and reducing power, and *pck*, encoding pyruvate carboxy kinase (Pck), was used to enhance the synthesis of oxalacetate through the carboxylation of phosphoenolpyruvate in an anaplerotic reaction. The phosphoenolpyruvate can subsequently react with the CO_2 and reducing equivalents to generate succinic acid. Plasmids pSBF2 (carrying *fdh1*) and pEcpck (carrying *pck*) were introduced into the Δ*cra* and parental strains. The coexpression of both Pck and Fdh1 led to an increase in succinate in both Δ*cra* and wild-type strains, but the increase was more marked in the mutant strain, achieving a difference of almost 100% in succinate concentration compared to the parental strain in nonaerated M9 cultures after 24 h (2.90 ± 0.60 g liter^{-1} and 1.40 ± 0.05 g liter^{-1}, respectively) (Fig. 5A). Induction of the enzymes did not cause significant changes in the metabolic profiles of the recombinants, apart from the increase in succinate synthesis (see Fig. S5 in the supplemental material).

A recent study tested different variants of Cra in a Δ*pfl* Δ*ldh E. coli* strain and resulted in a significant increase in succinate synthesis using a modified regulator that constitutively activates *aceBAK* (27). Although the modified Cra could affect metabolic fluxes differently from the absence of the regulator, the effect on succinate production of both kinds of manipulations supports the notion that *cra* is a key regulator that has an important effect on the synthesis of this added-value compound.

Enhancement of 1,3-PDO production in Δ*cra* and Δ*arcA* mutants with PhaP. The results described above indicated that the Δ*arcA* strain exhibited the best perfor-

mance for 1,3-PDO synthesis when cultured in rich medium and fully aerated conditions (Fig. 3). This mutant strain displayed increased yield and glycerol consumption under these conditions (Table 5) and a higher tolerance to 1,3-PDO than any of the other strains (Table 2). On the other hand, Δ*cra* mutants showed higher specific productivity of 1,3-PDO compared to the parental strain (Table 4). Since the Δ*cra* strain has been shown to have a reduced ability to grow on gluconeogenic carbon sources such as glycerol in minimal medium (8), we hypothesized that this substrate could be funneled into 1,3-PDO rather than into biomass when grown on a rich medium. However, when LB cultures of this strain were conducted, only a slight increase in 1,3-PDO was detected (Fig. 3). Since Δ*cra* derivatives exhibited an increased sensitivity to most of the stress agents tested, slight increases in 1,3-PDO could be due to an increased sensitivity to stressing culture conditions or fermentation inhibitors, which could prevent diol production.

Several different approaches have been used to overcome stress and tolerance problems, including evolution experiments in which resistant strains are selected by increasing amounts of the bioproduct (48), as well as other approaches. For example, the expression of chaperones has been used to enhance bacterial tolerance to diverse compounds, such as EtOH, in several strains (49). Previous studies in our laboratory have demonstrated the protective effect of PhaP, a PHB granule-associated protein from *Azotobacter* sp. FA8, against several types of stress (50). More recently, PhaP was shown to improve tolerance to biofuels and enhance EtOH and 1,3-PDO production in *E. coli* (51). In view of the results obtained in other *E. coli* strains, PhaP was overexpressed in the Δ*arcA* and Δ*cra* mutants of the Keio collection to test its ability to enhance 1,3-PDO production.

Synthesis of 1,3-PDO was determined in fully aerated LB cultures of *E. coli* BW25113 and the Δ*arcA* and Δ*cra* variants transformed with plasmid pPhaP328, harboring *phaP* under the control of the Pm promoter. Although overexpression of *phaP* caused a significant increase in diol production in the Δ*cra* strain, this effect was unexpectedly less important than the effect observed in the wild-type background (Fig. 5B). This could indicate that, despite the increased sensitivity of the Δ*cra* mutants to many stressors, the poor performance for 1,3-PDO synthesis could be related to other factors. These results suggest that this global regulator mutant would not be adequate for diol synthesis. In the case of the Δ*arcA* mutant, overexpression of PhaP resulted in a significant increase in 1,3-PDO production, both in comparison to the mutant strain without PhaP and also in comparison to the wild type with PhaP, resulting in a 2.3-fold increase. These data indicate that the beneficial effects of PhaP enhanced the metabolic effects of the Δ*arcA* deletion further, achieving an even higher increase in the production of the diol from glycerol. The final amount of 1,3-PDO produced by the Δ*arcA* strain in the presence of PhaP was 17 ± 1 g liter^{-1}, with a yield of 0.73 ± 0.01 mol · mol^{-1} (Fig. 5B and Table 5).

Taking into account the promising results obtained with the Δ*arcA* mutant with PhaP, the behavior of this strain was analyzed under conditions more similar to those encountered in bioprocesses. Our first approach was to use a semidefined medium (SDM) more compatible with future applications. Shaken-flask cultures were grown in a modified M9 medium supplemented with 5 g liter^{-1} of yeast extract and 30 g liter^{-1} of glycerol. Only a slight reduction in diol titers was noticed for both strains expressing PhaP compared to LB cultures, and the Δ*arcA* strain again showed a remarkable increase in 1,3-PDO (1.84-fold) compared to the wild-type strain (Fig. 5B).

Lastly, repeated batch cultures of the wild-type and Δ*arcA* strains were grown in a bioreactor for 48 h in SDM. The glycerol concentration was restored to 30 g liter^{-1} when it reached levels lower than 5 g liter^{-1}. The wild-type strain reached 11 g liter^{-1} of 1,3-PDO with a volumetric productivity of 0.24 ± 0.02 g liter^{-1} h^{-1}, while the Δ*arcA* culture produced 73% more 1,3-PDO, achieving 19 g liter^{-1} and a productivity of 0.38 ± 0.01 g liter^{-1} h^{-1} (Fig. 6A and Table 6).

Since *arcA* mutants are known to provide a reduced intracellular environment, some previous studies have suggested the potential use of these mutants for 1,3-PDO

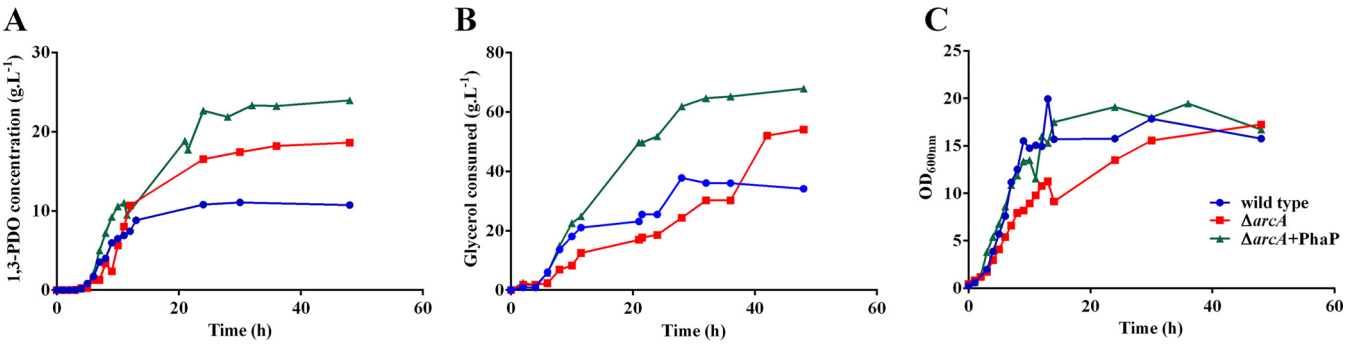

FIG 6 Production of 1,3-PDO in Δ*arcA* mutants and effect of PhaP on diol synthesis. Cultures of *E. coli* BW25113, its Δ*arcA* derivative containing plasmid pBBR1MCS-2::*dha* (wild type and Δ*arcA* strains), and a Δ*arcA* mutant containing both plasmids pBBR1MCS-2::*dha* and pPhaP328 (Δ*arcA*+PhaP strain) were grown in SDM supplemented with glycerol at 30 g liter^{-1} in a bioreactor under fully aerated conditions for 48 h. The expression of *phaP* was induced by the addition of 0.5 mM *m*-toluic acid when cultures reached the early exponential phase (OD_{600} = 0.8). Representative data from two independent fermentations are presented. (A) 1,3-PDO production; (B) glycerol consumed; (C) growth. Plasmid pBBR1MCS-2::*dha* contains the *dha* operon from *K. pneumoniae*; plasmid pPhaP328 contains *phaP* from *Azotobacter* sp. FA8.

synthesis. However, deletion of this global regulator in *Klebsiella pneumoniae* (52) or in *E. coli* (53) led to only slight increases (between 6 and 16.7%) in the synthesis of 1,3-PDO, while in *Citrobacter freundii* the *arcA* deletion did not affect 1,3-PDO yield (54). In all these cases, additional mutations and genetic modifications were used during the development of overproducing strains, masking the effect of the *arcA* deletion. In contrast, a 73% increase in 1,3-PDO synthesis was obtained in the present work in a Δ*arcA* mutant containing no additional mutations. This highlights the importance of using a well-characterized *E. coli* strain in order to single out the particular effects of the global regulator manipulations.

When PhaP was expressed in the Δ*arcA* genetic background, the repeated batch cultures reached 24 g liter^{-1} of 1,3-PDO, which represents an increase of 2.2-fold compared to the wild type after 48 h (Fig. 6A). The final biomasses were similar for all strains, although the Δ*arcA* mutant showed a reduced growth rate compared to the wild-type strain (Fig. 6C) that was restored by the overexpression of *phaP* (Table 6). The wild-type strain ceased both 1,3-PDO synthesis and glycerol consumption after 24 h, while both Δ*arcA* mutants continued using glycerol to synthesize 1,3-PDO for more than 40 h. Cultures of the Δ*arcA* strain with PhaP consumed glycerol faster, which also increased the rate of 1,3-PDO synthesis, resulting in a higher productivity (0.53 ± 0.02 g liter^{-1} h^{-1}) (Fig. 6B and Table 6). In view of these results, it can be hypothesized that the absence of ArcA caused the cells to continue using the substrate, since this was observed both with and without PhaP, while the presence of PhaP allowed the cells to grow faster and convert more glycerol into 1,3-PDO, an effect that was previously reported in other *E. coli* strains (50). The joint effects of these two manipulations allowed a very important increase in the production of 1,3-PDO, since the growth-

TABLE 6 Production of 1,3-propanediol in repeated batch fermentation[a]

Strain	μ_{max}[b] (h^{-1})	Yield (mol 1,3-PDO · mol Gly^{-1})	Volumetric productivity (g · liter^{-1} h^{-1})	Specific productivity (g · liter^{-1} g CDW^{-1} h^{-1})	Glycerol uptake[c] (g · liter^{-1} g CDW^{-1} h^{-1})
Wild type	0.39 ± 0.02	0.40 ± 0.06	0.24 ± 0.02	0.06 ± 0.01	0.18 ± 0.01
Δ*arcA* mutant	0.27 ± 0.02	0.41 ± 0.04	0.38 ± 0.01	0.08 ± 0.01	0.26 ± 0.03
Δ*arcA*+PhaP	0.34 ± 0.01	0.42 ± 0.01	0.53 ± 0.02	0.11 ± 0.01	0.31 ± 0.02

[a]Cultures were grown in semidefined medium supplemented with glycerol at 30 g · liter^{-1} in a bioreactor under fully aerated conditions for 48 h. Results represent means ± the standard deviations of duplicate measurements from at least two independent cultures. The strains used were *E. coli* BW25113 as a control (wild type) and its Δ*arcA* derivative. All strains harbored plasmid pBBR1MCS-2::*dha* (carrying the genes for 1,3-PDO synthesis). Both Δ*arcA* strains carry, additionally, pPhaP328 (carrying *phaP* from *Azotobacter* sp. FA8). In the Δ*arcA*+PhaP strain, the expression of *phaP* was induced by the addition of 0.5 mM *m*-toluic acid when the cultures reached the early exponential phase (OD_{600} = 0.8). Gly, glycerol; CDW, cell dry weight.
[b]Maximum specific growth rate.
[c]Specific glycerol uptake rate.

TABLE 7 Strains and plasmids

Strain, plasmid, or primer	Relevant characteristics or sequence (5′-3′)[a]	Source or reference[b]
E. coli strains		
BW25113	F$^-$ Δ(*araD-araB*)567 Δ*lacZ4787*(::*rrnB-3*) λ$^-$ *rph-1* Δ(*rhaD-rhaB*)568 *hsdR514*	CGSC (31)
Δ*rob* mutant	Same as BW25113, but Δ*rob*-721::*kan*	CGSC (31)
Δ*cra* mutant	Same as BW25113, but Δ*fruR*786::*kan*	CGSC (31)
Δ*arcA* mutant	Same as BW25113, but Δ*arcA*726::*kan*	CGSC (31)
Δ*creC* mutant	Same as BW25113, but Δ*creC*724::*kan*	CGSC (31)
Plasmids		
pCP20	Plasmid used for *kan* excision; *Saccharomyces cerevisiae* FLP λ cI857 λPR *repA*(Ts); Ap^r Cm^r	20
pSBF2	Plasmid pDHK30 carrying *fdh* from *Candida boidinii* under the control of the *lac* promoter; Km^r	64
pEcPck	Plasmid pTrc99A(1) carrying *pck* from *E. coli* under the control of the *lac* promoter; Ap^r	63
pET$_{Lm}$	Plasmid pBluescript II KS(−) carrying *adhE* from *Leuconostoc mesenteroides*; Ap^r	51
pBBR1MCS-2::*dha*	Plasmid pBBR1MCS-2 carrying *dhaR, dhaG, dhaT, dhaB, dhaC, dhaE*, and *dhaF* from *Klebsiella pneumoniae* GLC29; Km^r	41, 62
pPHB-KF	Plasmid pJP24 (58) carrying the *phaCAB* genes from *Ralstonia eutropha* (57) and *kan*-FRT gene from plasmid pKD4 (59); Ap^r Km^r	This study
pSEVA328	Broad-host-range vector; *oriRK2*, *oriT*; Cm^r	61
pPhaP328	Plasmid SEVA328 carrying the *phaP* gene from *Azotobacter* sp. strain FA8 under the control of the Pm promoter; Cm^r	This study
Primers		
PhaP-F	ATT GGA TCC ACA CAG GAA ACA GCT ATG GCT TTT TTT GAT CTG	This study
PhaPLow	TCG AAG CTT GCC GTC AGG CAG TCT T	60

[a]Antibiotic resistance markers: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin.
[b]CGSC, Coli Genetic Stock Center (Yale University, New Haven, CT).

enhancing effect of PhaP boosted the beneficial metabolic background generated by the *arcA* deletion even further.

Conclusion. The combined analysis of multiple variables showed specific patterns for each global regulator mutation in *E. coli* BW25113 despite the extensive regulation exerted by the transcription factors and their coregulation of some gene targets (3, 44, 45). It is important to highlight that (i) the genome of this strain has been completely sequenced and does not contain additional mutations (55) that could mask or interfere with the effects of the regulators and that (ii) analyzing the different mutations in a uniform genetic background eliminates possible strain-dependent results. This approach enabled the characterization of the physiological consequences of altered carbon and redox fluxes driven by the global regulator deletions and singled out Cra and ArcA as the regulators with the most important effects on bacterial metabolism. Using the production of succinate and 1,3-PDO as a proof of concept, Δ*cra* and Δ*arcA* mutants were subjected to further manipulations to obtain larger amounts of these compounds, demonstrating that the metabolic backgrounds of the mutants were suitable for the synthesis of bioproducts.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* BW25113 and its derivatives were obtained from Coli Genetic Stock Center (Yale University, New Haven, CT). Strains and plasmids are listed in Table 7. All mutant strains were transformed with plasmid pCP20 for *kan* cassette resistance removal (56), and the plasmid was eliminated by consecutive passages of bacteria in LB plates at 42°C. Excision of the *kan* cassette was confirmed by PCR.

Construction of pPHB-KF and pPhaP328. An EcoRI-BamHI fragment from plasmid pTZ18U-PHB containing the *phaCAB* genes from *Ralstonia eutropha* (57) was ligated into plasmid pJP24 (58). A fragment containing the removable *kan*-FRT gene from plasmid pKD4 (59) was amplified by PCR and ligated into pJP24 as well. Plasmid pADP2 was used as the template for PCR amplification of a BamHI-HindIII fragment containing *phaP* from *Azotobacter* sp. FA8 (60). The PCR product was ligated to pSEVA328 (61) to obtain pPhaP328, which carries *phaP* under the control of the Pm promoter. Plasmid constructions were checked by PCR and agarose gel electrophoresis.

Growth media and culture conditions. (i) Metabolic profiles and tolerance assays. M9 minimal medium (20) supplemented with glucose at 5 g liter^{-1} was used. The aeration conditions were established by a combination of agitation and the relationship between the volume of culture medium

(V_m) and the volume of the flask (V_f) as follows: for full aeration, 200 rpm and $V_m/V_f = 1/10$; and for low aeration, 125 rpm and $V_m/V_f = 1/2$ (20). Penicillin bottles (50 ml) capped with cotton plugs and 10-ml glass vials covered with rubber caps were used for fully aerated and low-aeration conditions, respectively. For tolerance assays, concentrations of each compound (as indicated in Fig. 2 and the footnote for Table 2) were added to the medium before inoculation. In the case of volatile compounds (EtOH and ButOH), vials were capped with rubber plugs and aluminum seals. Nonaerated cultures were grown in 10-ml screw-cap tubes filled with medium agitated at 6 rpm to avoid cell sedimentation.

Redox stress tests were performed in M9 agar plates with the addition of toluidine blue at a 0.02% final concentration. Serial dilutions from overnight cultures were spotted over the plates to obtain isolated colonies, and colony diameters were determined after 24 h at 37°C.

All cultures were inoculated from overnight precultures grown under the same conditions as working cultures. Precultures were started from frozen glycerol stocks plated on LB agar medium, and two to three colonies were picked for each case.

(ii) Production of chemicals in recombinant strains. For EtOH production, the low-aeration conditions described above were implemented for cultures grown in LB (containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) supplemented with 10 g of glucose or 10 g of glycerol per liter incubated at 37°C for 24 h. All mutant and wild-type strains were transformed with plasmid pET_{Lm} (50). The expression of *adhE* was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when an optical density at 600 nm (OD_{600}) of 0.8 was reached.

In the case of 1,3-PDO, strains transformed with plasmid pBBR1MCS-2::*dha* (41, 62) were grown in M9 minimal medium or LB under full- and low-aeration conditions. In all cases, medium was supplemented with glycerol (10 g $liter^{-1}$ and 30 g $liter^{-1}$ were added for low and full aeration, respectively) and 1.5 μM vitamin B_{12}. When pPhaP328 was used, *phaP* expression was induced with 0.5 mM *m*-toluic acid added at an OD_{600} of 0.8. PHB-producing strains transformed with the pPHB-KF plasmid were grown in LB medium with glucose 10 g $liter^{-1}$ under low-aeration conditions in 100-ml cylinder flask bottles. Antibiotics (kanamycin, 50 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}) were added whenever needed.

(iii) Cultures of engineered Δ*cra* mutant for enhanced succinic acid production. Wild-type and Δ*cra* strains, transformed with pEcpck (63) and pSBF2 (64), were grown under nonaerated conditions as described above for 24 h in M9 medium with the addition of 0.5 g $liter^{-1}$ of yeast extract, 5 μg $liter^{-1}$ of thiamine, and trace solution (containing [per liter] 2.78 mg of $FeSO_4 \cdot 7H_2O$, 1.98 mg of $MnCl_2 \cdot 4H_2O$, 2.81 mg of $CoSO_4 \cdot 7H_2O$, 0.17 mg of $CuCl_2 \cdot 2H_2O$, and 0.29 mg of $ZnSO_4 \cdot 7H_2O$ in 1 N HCl). In this case, a low-aeration preculture step was added before the nonaerated preculture to obtain larger amounts of biomass. Expression of *fdh1* and *pck* was induced by the addition of 0.5 mM IPTG at an OD_{600} of 0.8. The culture medium also contained 100 mM $NaHCO_3$ as a CO_2 source.

(iv) 1,3-PDO fermentation conditions (repeated batch). LB precultures grown in Erlenmeyer flasks containing 75 ml of medium under the fully aerated conditions defined above were used to inoculate a 3.7-liter bioengineering bioreactor filled with 1.5 liters of M9 medium containing (per liter) 6.0 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl, 2.0 g of NH_4Cl, 0.4 g of $MgSO_4$, 0.01 g of $CaCl_2$, and 0.06 g of ammonium iron(III) citrate. Next, 30 g $liter^{-1}$ of glycerol, 5 g $liter^{-1}$ of yeast extract, and 1.5 μM vitamin B_{12} and kanamycin were added. The pH was adjusted to 7 by the addition of 2 M NaOH and 1 M H_2SO_4. Dissolved oxygen was maintained at 20% during the whole cultivation by a cascade mode, and the air supply was set at between 0.5 and 1.5 liters min^{-1}. The OD, biomass, and 1,3-PDO and glycerol titers were monitored during the 48-h fermentation. The glycerol concentration was restored to 30 g $liter^{-1}$ when it reached levels below 5 g $liter^{-1}$ in the culture supernatant.

Analytical determinations. (i) Organic acid determination. Cultures were centrifuged at 5,000 rpm for 6 min, and supernatants were diluted 1:3 and 1:5 in water for the low-aeration and nonaerated conditions, respectively. The cell pellets obtained were dried at 65°C for 48 h for cell dry weight (CDW) measurements. Filtered (0.22-μm pore size) liquid samples were analyzed by high-pressure liquid chromatography (LC-20AT Prominence; Shimadzu Corp., Kyoto, Japan) equipped with a UV detector (SPD-20AV; Shimadzu Corp.) using an Aminex HTX-87H column (catalog no. 170 0140; Bio-Rad Laboratories, Inc., Hercules, CA). The mobile phase was 5 mM H_2SO_4 in water, and the flux rate was 0.6 ml min^{-1} at 50°C. Quantification was done at 215 nm, and analytical standards (Sigma-Aldrich Co., St. Louis, MO) were used.

(ii) EtOH, 1,3-PDO, and glycerol determinations. Sample preparations and compound measurements were performed as previously indicated (50, 65). Briefly, samples were centrifuged and filtered as indicated for organic acids (see the previous paragraph), and 250 μl of each sample was diluted in 750 μl of EtOH or acetone for the 1,3-PDO or EtOH determination, respectively. Finally, the dilutions were vortexed for 1 min and centrifuged at 9,000 rpm and 3 min for salt precipitation. Gas chromatography (GC) was carried out using a 7820A chromatographic system equipped with a flame ionization detector (FID) and an automatic liquid sampler ALS 7693 (Agilent, Santa Clara, CA). HP-INNOWAX capillary column (30 m; 0.25-μm film thickness, and 0.25-mm internal diameter) was used with N_2 as the carrier gas. Briefly, for glycol measurement, the GC oven was heated at 185°C for 3 min, then ramped up to 220°C at 40°C min^{-1}, and held for 1 min. The injector and FID temperatures were set at 290 and 300°C, respectively, and the gas column flow was 2.5 ml min^{-1}. For EtOH, the oven temperature was set at 35°C for 8 min. The injector and detector temperatures were 150 and 300°C, respectively. The nitrogen flow was 1 ml min^{-1}. The split ratios were 30:1 and 90:1 for glycols and EtOH, respectively.

(iii) Glucose determination. An enzymatic kit (Wiener Laboratorios SAIC, Rosario, Argentina) was utilized, and samples were previously centrifuged and diluted 1:5 in deionized water. The colorimetric reaction was measured with a UV-Vis spectrometer set at 505 nm.

(iv) PHB determination. The cultures were centrifuged, and the supernatants were discarded. The amount of polymer was determined by GC as previously described (17). Pellets were lyophilized overnight, and 10-mg portions were placed in Teflon screw-cup vials. The pellets were heated at 100°C for 2 h with 2 ml of chloroform and 2 ml of 15% (vol/vol) sulfuric acid diluted in methanol. After cooling at room temperature, the aqueous phase was discarded, and the samples were washed twice with 2 ml of deionized water and dried over anhydrous Na_2SO_4. The resulting methyl esters of 3-hydroxybutyrate were quantified in the chromatographic system described above. An HP-5 nonpolar column was used (30 m; 0.25-μm film thickness, 0.32-mm internal diameter). The GC oven was initially heated at 60°C for 3 min and then to 110°C with a heat ramp of 45°C min^{-1} and finally held for 1.5 min. The injector and FID were set to 250 and 300°C, respectively. The split ratio was 45:1, and 1 μl was injected.

(v) Statistical analysis. All of the experiments reported here were performed at least twice in a minimum of two independently grown cultures. The mean values of the corresponding parameter $\pm$ the standard deviations of triplicates for each assay are presented. The statistical significance of differences when comparing two strains was obtained using a two-tailed Student *t* test. Data were considered statistically significant when the *P* value was <0.05. In the case of bioreactor cultures, representative data from at least two independent cultures are shown.

Multivariate descriptive analysis was performed by unsupervised principal component analysis (PCA), discriminant analysis (DA), and hierarchical clustering analysis (HCA) using InfoStat Software. These tools were used to identify separation trends and to visualize the response of the working strains under the variety of conditions tested. A total of 21 variables were used in the statistical analysis, including tolerance assays, metabolic profiles, and the biomass yields of the strains in minimal medium. HCA was calculated using Euclidean distance approach, and the five groups were clustered using Ward's method.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01337-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

We are indebted to G. N. Bennett (Rice University, Houston, TX) and to P. Kim (Catholic University of Korea, Bucheon, Gyunggi, South Korea) for the kind gifts of plasmids pSBF2 and pEcPck, respectively. We are grateful to J. G. Cabrera Gomez and to A. Flora for providing plasmid pBBR1MCS-2::*dha*, to V. de Lorenzo for providing plasmid pSEVA328, and to P. I. Nikel for plasmid pET_{Lm}.

M.J.P. and M.P.M. are career investigators from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). D.E.E., R.D.P., and D.S.A. hold doctoral fellowships from CONICET.

We declare no conflict of interest.

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