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Escherichia coli FocA/B-dependent H^+ and K^+ fluxes: Influence of exogenous versus endogenous formate

L. Grigoryan,[1,2,3] A. Babayan,[1,2,3] A. Vassilian,[2,4,*] A. Poladyan,[1,2,*] G. Sawers,[5] and K. Trchounian[1,2,3,*]

[1]Department of Biochemistry, Microbiology and Biotechnology, Faculty of Biology, Yerevan State University, Yerevan, Armenia; [2]Research Institute of Biology, Yerevan State University, Yerevan, Armenia; [3]Microbial Biotechnologies and Biofuel Innovation Center, Yerevan State University, Yerevan, Armenia; [4]Department of Ecology and Nature Protection, Faculty of Biology, Yerevan State University, Yerevan, Armenia; and [5]Institute of Microbiology, Martin-Luther University of Halle-Wittenberg, Halle (Saale), Germany

ABSTRACT *Escherichia coli* translocates formate/formic acid bidirectionally across the cytoplasmic membrane by the FocA/FocB formate channels during fermentation. Depending on the pH and whether formate is supplied exogenously or generated internally, the mechanisms of translocation differ. This study elucidates the role of these channels in dependence on F_OF_1 ATPase activity in stationary phase cells after cultivation by mixed-carbon fermentation at pH 7.5. In cells cultivated with glucose plus glycerol, exogenously added formate increased the *N,N′*-dicyclohexylcarbodiimide (DCCD)-sensitive (F_OF_1 ATPase-dependent) proton flux in single or double *foc* mutants. Moreover, exogenously supplied formate also increased the DCCD-sensitive potassium flux, but only in mutants where *focB* was absent. In the cells grown on glucose, glycerol, and formate, addition of formate in the whole-cell assays increased F_OF_1 ATPase activity by ∼60% compared with cells grown on a mixture of only glucose and glycerol. In a *focA* mutant cultivated to the stationary phase on glucose, glycerol, and formate, F_OF_1 ATPase activity was double that compared with cells grown on only glucose and glycerol, while in a *focA-focB* double-null mutant F_OF_1 ATPase activity decreased by ∼50% in formate assays. These data suggest that the cell regulates the mechanism of formate translocation depending on whether formate is generated internally or added exogenously. Thus, F_OF_1-ATPase activity and the FocA/FocB channels together with formate hydrogenlyase activity combine to balance pH and ion gradients during fermentation in stationary phase cells in response to whether formate is generated metabolically or supplied in high concentration from the environment.

WHY IT MATTERS It is important to understand the bacterial cell homeostatic mechanisms when fermenting cells are in the stationary phase. Formate channels, F_OF_1-ATPase, and potassium- and proton-transporting systems all contribute to ion homeostasis during fermentation. The role of the FocA/B formate channels was investigated in the presence of exogenously supplied or endogenously produced formate. We show that a functional link between FocA and F_OF_1-ATPase activity appears to depend on the source of formate. FocA plays an important role in proton transport and FocB is crucial for regulating potassium transport, and these all depend on an active F_OF_1-ATPase. We find that in stationary phase fermenting cells the balance of pH and ion gradients crucially depend on F_OF_1-ATPase and the formate channels.

INTRODUCTION

Coordinating regulation of formate metabolism with proton ion gradients in *Escherichia coli* could provide a mechanism for efficient energy conservation and pH homeostasis during fermentation, particularly in the stationary phase. Formate activates various pathways to help maintain a stable transmembrane proton gradient (1–3). Besides formate, proton translocation also plays a major role in balancing the pH and ion gradients across the cytoplasmic membrane of the cell by H^+/H_2 cycling, whereby hydrogenases also play an important role (3). It is also well known that transport of protons via proton F_OF_1-ATPase is coupled to the synthesis or hydrolysis of ATP (4). During fermentation, F_OF_1-ATPase functions in ATP hydrolysis mode, where protons are transported out of the cell. In this regard, it has been recently proposed

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*Correspondence: anaitvassilian@ysu.am or apoladyan@ysu.am or k.trchounian@ysu.am

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that, during aerobic respiration in mitochondria, the movement of H^+ across biological membranes occurs via interactions with specific charged sites or regions within the membrane, which is referred to as the transmembrane-electrostatically localized proton (TELP) theory and has been proposed as a complement to Mitchell's chemiosmotic theory (5–7). TELP theory offers a molecular-level understanding of how protons potentially move across membranes, particularly in the context of proton pumps and transporters (5,6), possibly also involving specific channel proteins.

It has been shown that, during glucose fermentation, *E. coli* produces formate inside the cell, which is first translocated out and then reimported into the cell when the external pH decreases (1). *E. coli* has two known formate channels, FocA and FocB, which are encoded by the *focA* and *focB* genes, respectively. For FocA it has been shown to function bidirectionally (8), while less is known about the role of FocB. Nevertheless, it has been suggested that, during mixed-carbon fermentation, FocB might function in formate import in stationary phase cells (9).

While the *focB* gene is located at the end of *hyf* operon, which encodes hydrogenase 4 (10), *focA* is cotranscribed with the gene encoding pyruvate formate-lyase (PflB) (11). In particular, synthesis of FocA and PflB is coordinated to regulate the generation and further metabolism of formate in the cell (12). Moreover, the pH-dependent uptake of formate by FocA has been shown to rely on a highly conserved histidine residue, His-209, within the pore of FocA (13).

An important feature of formate metabolism is the maintenance of balanced intracellular formate concentration, particularly in relation to internal and external pH. During fermentative growth with glucose, this is controlled not only by FocA, but also by the formate hydrogenlyase (FHL-1) complex, which disproportionates formic acid into H_2 and CO_2 (14). This helps to balance intracellular as well as extracellular pH levels. Notably, Metcalfe et al. (14) showed that, in the presence of high concentrations of exogenous formate, intracellular generation of formate by PflB continued while the exogenously supplied formate was imported and directly dispropotionated to H_2 and CO_2 by FHL-1. This activity helped to offset acidification of the immediate cellular environment.

It has also been shown using stationary phase *E. coli* cells that exogenously added formate causes proton efflux, which is dependent on the activity of F_OF_1-ATPase (8). In contrast, in an *E. coli* mutant synthesizing a $FocA_{H209N}$ variant that results in a formate-efflux phenotype, the proton flux out of the cell is increased significantly (8). The relationship between F_OF_1-ATPase and formate translocation via FocA thus appears to contribute to the maintenance of the pH gradient and possibly to proton motive force generation. In addition, formate translocation by FocA also contributes to ion homeostasis by influencing potassium transport (15). During fermentative growth of *E. coli* on a mixture of glucose and glycerol or glucose, glycerol, and formate, it has been shown that formate is taken up into the cell mainly by FocA at pH 7.5, while FocB appears not to be involved in formate uptake under these conditions (16).

A metabolic link between F_OF_1-ATPase activity and that of secondary transport systems during fermentation was originally suggested around 20 years ago (17). Recent experimental evidence has shown that, in the absence of F_OF_1-ATPase, either through mutation or inhibition of its activity by *N,N′*-dicyclohexylcarbodiimide (DCCD), influences not only potassium transport but also hydrogenase activity, succinate transport (18), and formate translocation by FocA and FocB (5–7). During fermentation of glucose as sole carbon and energy source, *E. coli* exchanges $2H^+$ for $1K^+$, but in cells subjected to osmotic stress this ratio is disturbed (17). In particular, during glucose fermentation at pH 7.5, FocB contributes significantly to the regulation of proton flux when cells are subjected to osmotic stress (15). Moreover, the addition of exogenous formate to the growth medium during osmotic stress conditions causes restoration of H_2 production by the FHL-1-dependent hydrogenase 3 (Hyd-3) enzyme, but not by Hyd-4, which associates with a second FHL-2 complex in *E. coli* (10,19).

In this study, we describe how the external and internal formate concentration influences *E. coli* metabolism during mixed-carbon source fermentation and reveal a possible coupling between of the FocA/FocB formate channels with F_OF_1-ATPase activity, which is suggested to regulate the proton motive force in response to formate levels.

MATERIALS AND METHODS

Bacterial strains and growth

The *E. coli* BW25113 or MC4100 (wild-type parental strains) and the *focA* and *focB* single and double mutants used in this study are listed in Table 1.

Bacteria from overnight cultures were transferred into potassium phosphate-buffered liquid peptone medium (pH 7.5, 20 g L^{-1} peptone, 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl; pH 5.5, 20 g L^{-1} peptone, 1.08 g L^{-1} K_2HPO_4, 15 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl) with 2 g L^{-1} glucose and 10 g L^{-1} glycerol (M1 condition), or 2 g L^{-1} glucose, 10 g L^{-1} glycerol, and 0.68 g L^{-1} sodium formate (M2 condition) as carbon sources. Bacteria were grown under anaerobic fermentative conditions at 37°C for 20–24 h until stationary phase was reached, as described (8,16). Immediately before the cells were harvested, the pH of the medium had decreased to

TABLE 1 Characteristics of the *E. coli* strains used in this study

Strains	Genotype	Absent or defective gene product	Source and/or reference
BW25113	*lacI*^{q} *rrnB*$_{T14}$*ΔlacZ*$_{W116}$ *hsdR514 ΔaraBAD*$_{AH33}$ *Δrha BAD*$_{LD78}$	wild-type	Baba et al. (20)
JW2477	BW25113 *ΔfocB*	formate channel B	Baba et al. (20)
MC4100	F^{-} *araD139 D(argF-lac)U169 ptsF25 deoC1 relA1 flbB530 rpsL150 L2*	wild-type	Roncero and Casadaban (21)
REK701	MC4100 *ΔfocA* (*focA* codons 114 and 115 changed to UAG and UAA, respectively)	formate channel A	Hakobyan et al. (9)
REK701ΔfocB	REK701 *ΔfocB*	formate channel A and B	Hakobyan et al. (9)

between pH 6.8 and 6.9. The pH was determined using a pH meter with a selective pH electrode (H1131B, Hanna Instruments, Woonsocket, Rhode Island) and adjustments were made using 0.1 M NaOH or 0.1 M HCl.

Measurement of H^+ and K^+ fluxes

H^+ and K^+ fluxes in washed, whole-cell suspensions were determined in the assays using Tris-phosphate buffer, pH 7.5 (150 mM Tris-phosphate containing 0.4 mM $MgSO_4$, 1 mM NaCl, 1 mM KCl) and upon addition of either 2 g L^{-1} glucose or 0.68 g L^{-1} formate to the bacterial suspension. Changes in H^+ (ΔJ_{H+}) or K^+ (ΔJ_{K+}) fluxes in the external medium were measured using selective pH (HI1131B, Hanna Instruments, Portugal) or potassium electrodes (HI4114, Hanna Instruments, Portugal) (8). The electrode readings were calibrated by titration of the medium with 0.01 M HCl or with 0.1 M KCl. ΔJ_{H+} and ΔJ_{K+} were expressed in mmol min^{-1} per 10^9 cells. Representative progress curves for the proton and potassium fluxes in *E. coli* during addition of glucose as electron donor are shown in Fig. S1. The direction of the ion fluxes is measured in the experimental assay and if the ion concentration increases (e.g., for H^+) in the medium, this shows that the ion is secreted out of the cell, and when the ion concentration decreases (e.g., K^+) in the experimental assay, the ion is taken up by the cells. The whole cells were incubated with 0.2 mM DCCD where indicated, as described (18). Note that both parental strains (BW25113 and MC4100) used in this study showed indistinguishable results in the experiments conducted during this study and consequently in the text, tables, and figures they are mentioned simply as wild-type.

Membrane vesicles and proton ATPase activity

Membrane vesicles (MVs) were obtained from bacteria by inducing the osmotic lysis of spheroplasts through treatment with lysozyme and ethylenediaminetetraacetic acid (22).

The total and DCCD-sensitive ATPase activities were determined by measuring the amount of inorganic phosphate (P_i) released in the reaction of MVs upon addition of 5 mM ATP. The reactions were conducted in 50 mM Tris-HCl buffer at 37°C, with the pH adjusted to match the respective growing environment (pH 7.5). ATPase activity was quantified in nmol P_i min^{-1} μg $protein^{-1}$. P_i was determined spectrophotometrically (UV-vis spectrophotometer, Cary 60, Agilent Technologies, Santa Clara, California) as described (16).

DCCD was used at a final concentration of 0.1 mM to determine the contribution of the F_OF_1-ATPase to total activity ATPase activity. The F_OF_1-ATPase activity was calculated as a difference between activities in the absence and in the presence of the inhibitor. To study the effect of potassium ions or formate in the assays, 100 mM potassium chloride or 10 mM sodium formate was added where indicated. MVs were incubated with K^+ ions or formate for 10 min. All assays were done at 37°C. Protein levels were measured by the method of Lowry et al. (23) using bovine serum albumin, as a standard.

Determination of external and intracellular pH, transmembrane pH gradient

Extracellular pH (pH_{ex}) was measured via a pH meter with a selective pH electrode (HI1131B, Hanna Instruments). Intracellular pH (pH_{in}) was determined using 9-aminoacridine fluorescent dye (9-AA) (with excitation at 390 nm and emission at 460 nm) (24). 9-AA is distributed across the membrane according to ΔpH, the uptake of which by bacterial cells was determined from the quenching of fluorescence (Cary Eclipse, Agilent Technologies). ΔpH was calculated as the difference between pH_{in} and pH_{ex}, as described elsewhere (25).

Statistical analysis

Each data point shown was the average from cultures using triplicate biological replicates and is presented as mean ± SD. A p value of less than 0.05 was considered significant. Data were visualized using GraphPad Prism 8.0.2 software (GraphPad Software) (26). Significance ($p < 0.05$) was determined by two-way ANOVA and Tukey's multiple comparison test for all data, as described before (27).

RESULTS

Proton fluxes during mixed-carbon source fermentation

E. coli has been studied during fermentative growth in the presence of glucose as sole carbon source (28–30), while in nature not only single carbon sources, but also mixtures of different carbon sources are present (31). In this study, we mixed different carbon sources (sugar, alcohol, and organic acid) during fermentative conditions with the aim of understanding how *E. coli* cells regulate their energy metabolism in response to exogenously supplied formate and how changes in ion gradients, in particular through the activity of F_OF_1-ATPase, are affected.

E. coli cells were grown at pH 7.5 under two fermentative conditions—first in the presence of the mixture of glucose or glycerol (M1 condition), or second in the presence of the mixture of glucose, glycerol and formate (M2 condition). It was determined that

when wild-type cells (BW25113 or MC4100) were grown under M1 conditions and when in the assays performed with washed whole-cell glucose was added as electron donor, the total H^+ flux (J_{H+}) was 2.4 mmol min^{-1} (Fig. 1 *A*). Note that, under all conditions and experiments tested, similar data were obtained for both wild-type strains. For strain REK701 (*focA*), the total J_{H+} was reduced by 50% compared with the wild-type. In the *focB* knockout mutant J_{H+} increased ∼25% compared with wild-type, reaching ∼3.0 mmol min^{-1} (Fig. 1 *A*). The addition of DCCD lowered the total proton flux by inhibiting the F_OF_1-ATPase. DCCD-sensitive J_{H+} in all mutants was higher compared with wild-type, suggesting that the FocA and FocB formate channels influence the F_OF_1-ATPase-dependent proton flux (Fig. 1 *B*). In the *focA* single mutant, the contribution of F_OF_1-ATPase increased ∼2.2-fold, while in the *focB* single mutant it increased ∼3.4-fold.

In the *focA-focB* double mutant (REK701Δ*focB*), the total J_{H+} was similar to the results for those of the *focA* single mutant, REK701 (Fig. 1 *A*). When both channels are absent, cells cannot regulate their formate metabolism efficiently and are reliant on slow diffusion of neutral formic acid across the membrane, and thus the deletion of both channels disturbs the existing metabolic cross talk with F_OF_1-ATPase (1,3). Similar mechanisms of formate translocation have been suggested for the hyperthermophile *Thermococcus onnurineus* (32).

When exogenous formate was added to wild-type cells in the assays, the total proton flux (J_{H+}) was 0.53 mmol min^{-1}, while in all mutants it was significantly decreased (Fig. 1 *A*). In the *focA* mutant, REK701, the proton flux was undetectable when compared with the wild-type, while in the single *focB* or *focA-focB* double mutants the measured total J_{H+} proton fluxes were similar to each other, but were decreased ∼2.6-fold compared with the wild-type (Fig. 1 *A*). In wild-type cells, the measured DCCD-sensitive J_{H+} was reduced by ∼50% indicating that half of the measured proton flux was due to F_OF_1-ATPase (Fig. 1 *A*). In the *focB* mutant the data showed that the proton flux was fully dependent on F_OF_1-ATPase activity (Fig. 1 *A*). In formate assays (externally added formate) the involvement of F_OF_1-ATPase was similar to wild-type and the lowered proton flux that was detected was thus independent the F_OF_1-ATPase. This suggests that, when glucose is used as electron donor (endogenously produced formate), FocB might influence directly or indirectly proton transfer by F_OF_1-ATPase through coupling of external formate uptake with FHL-1 activity, and might contribute to other systems, which deal with intramembrane or transmembrane proton transport. This suggests proton flux and pH homeostasis in stationary phase fermenting cells might be aided by balancing formate/formic acid translocation through the FocA/B channels and FHL-1 complex-dependent formic acid disproportionation to H_2 and CO_2 and that these modulate the activity of the F_OF_1-ATPase (33).

In glycerol assays in wild-type cells J_{H+} was similar to that measure in assays in which formate was added as electron donor, reaching 0.52 mmol min^{-1}. In single *focA* and *focA-focB* double mutants the results were similar to each other, but lowered ∼3.5-fold compared with wild-type. In the *focB* single mutant, J_{H+} was 0.33 mmol min^{-1}. This suggests that there is different regulation of formate metabolism when glycerol is used as a fermentable substrate. DCCD did not affect J_{H+} in wild-type and mutant cells (Fig. 1, *A* and *B*).

When wild-type cells were grown anaerobically under M2 conditions and used in glucose assays, the total J_{H+} increased by ∼20%, reaching 3 mmol min^{-1}. When external formate was present in the growth medium, the behavior of the cells was different and the *focB* single mutant and *focA-focB* double mutant had similar values of ∼3.6 mmol min^{-1} (Fig. 2 *A*). More importantly, J_{H+} in the *focA* mutant decreased by ∼50% compared with wild-type, which is a similar effect to that observed after growth of the cells under M1 conditions. The data clearly show that the presence of exogenously added formate had a strong effect on proton flux. It was shown earlier that, when cells are grown under M2 conditions, the cells initially take up formate and then start to ferment glucose (16), which suggests that the amount of internal formate might be increased to balance the first hours of the cell's response to external formate in the medium. In contrast to what was observed for cultivation under M1 conditions, DCCD-sensitive J_{H+} was similar in all mutants except for the *focA-focB* double mutant (Fig. 2 *B*). When comparing the effect of external formate on the DCCD-sensitive J_{H+} in the *focA* mutant, a similar increase in DCCD-sensitive proton flux compared with that seen for cells grown under M1 conditions was observed. This suggests that the presence of exogenous formate changes the regulation of the proton balance in the cells.

In wild-type and mutant cells in the formate assays, the total J_{H+} was minimal compared with the cells grown on M1 conditions (Fig. 2, *A* and *B*).

In assays in which glycerol was electron donor, wild-type cells and the *focA* single mutant grown on M2 conditions showed only residual J_{H+}. In the *focB* single and the *focA-focB* double mutants J_{H+} reached to 0.2 mmol min^{-1}. DCCD addition in the assays did not change the flux, suggesting that, in this assay, F_OF_1-ATPase activity did not contribute to the total proton flux.

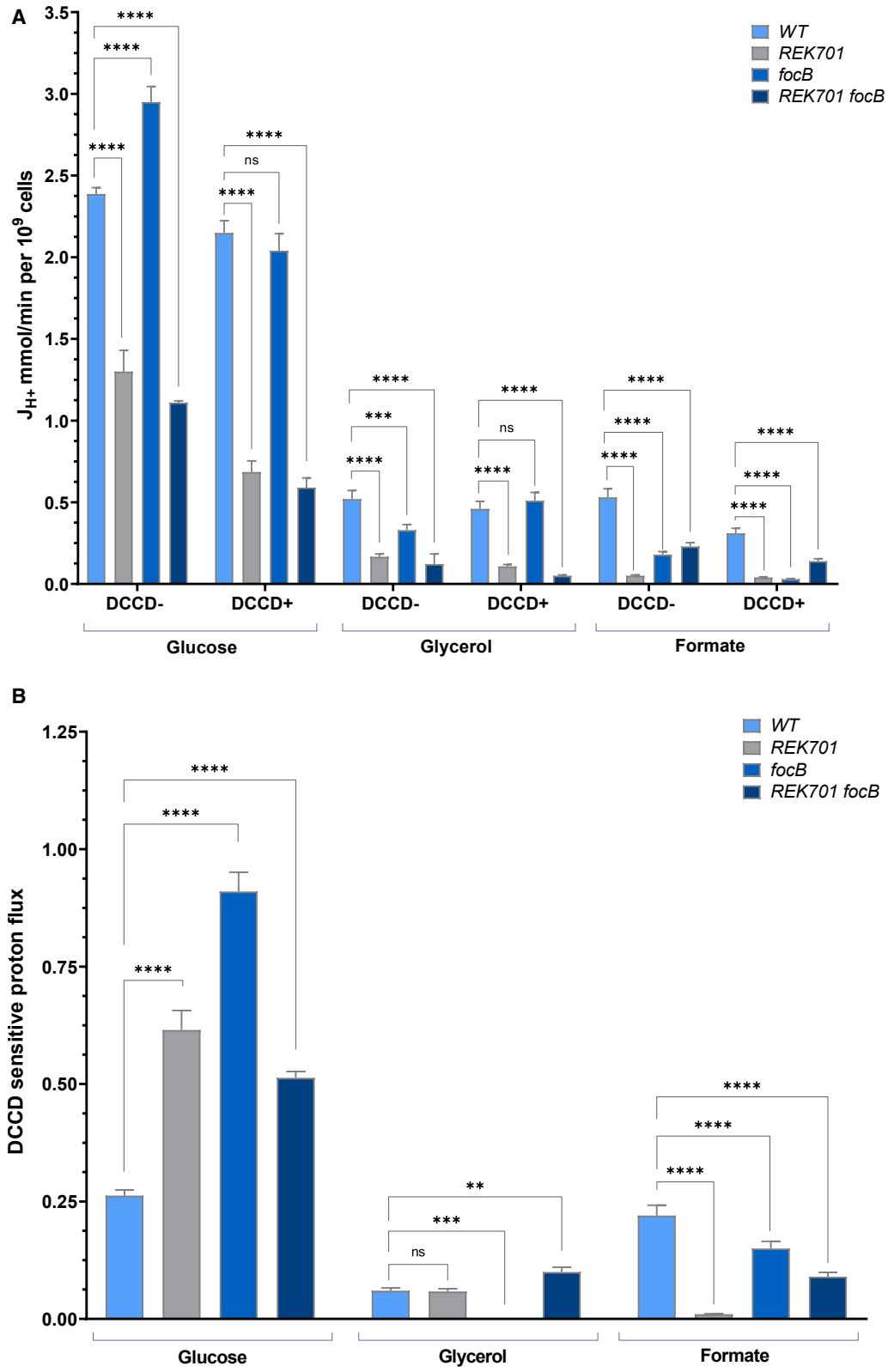

FIGURE 1 Determination of total (*A*) and DCCD-sensitive (*B*) H^+ flux by whole cells of *E. coli* wild-type and *foc* mutants after fermentative growth until the stationary phase with glucose and glycerol (M1 condition) at pH 7.5. Whole-cell assays were performed with either glucose (2 g L^{-1}), glycerol (10 g L^{-1}), or formate (0.68 g L^{-1}) as electron donor. DCCD (0.2 mM) was added to the assay medium where indicated. For others, see materials and methods.

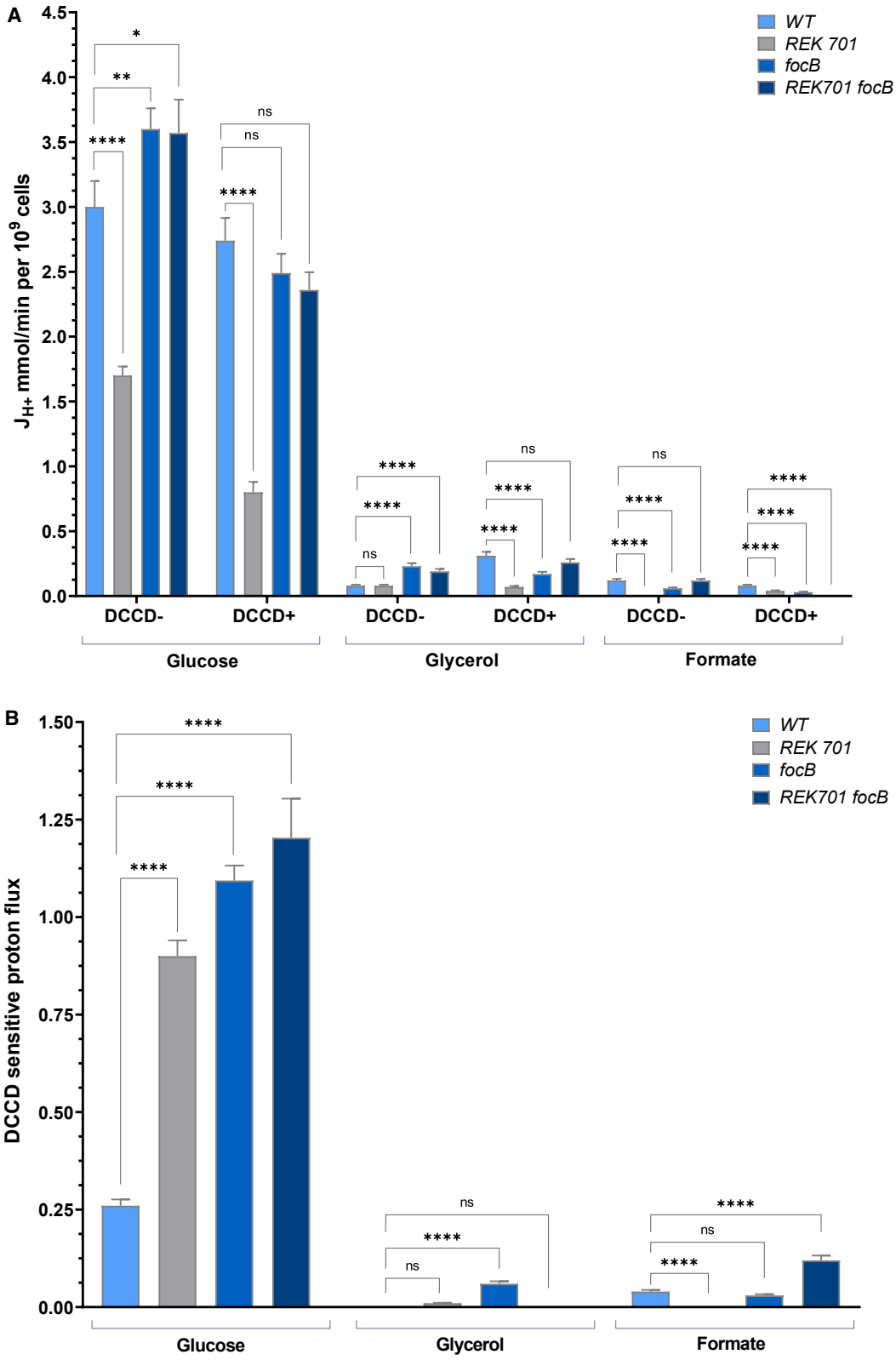

FIGURE 2 Total (*A*) and DCCD-sensitive (*B*) H^+ flux in whole cells of *E. coli* wild-type and *foc* mutants after fermentative growth until the stationary phase in the presence of glucose, glycerol, and formate (M2 condition) at pH 7.5. Assays were performed with whole cells and glucose (2 g L^{-1}), glycerol (10 g L^{-1}), or formate (0.68 g L^{-1}) was added as electron donor. DCCD (0.2 mM) was added to the assay medium where indicated.

Potassium fluxes during mixed-carbon source fermentation

One of the main ion gradients that *E. coli* cells must regulate and maintain is the potassium ion gradient (34), including systems that are linked to F_OF_1-ATPase activity and other secondary systems (17). It has been previously shown that the FocA and FocB channels contribute to the regulation of both proton and potassium fluxes in *E. coli* during glucose fermentation and they appear to aid cell survival under osmotic stress conditions (15). In this study, wild-type cells grown under M1 conditions in glucose-supplemented assays had a total J_{K+} of 0.22 mmol min^{-1} (Fig. 3 *A*). For all mutants, with the exception of the *focB* mutant, the total J_{K+} was similar to that measured for the wild-type. In the *focB* mutant the J_{K+} decreased ∼30% reaching a level of 0.16 mmol min^{-1}. This might suggest that FocB has an influence on the potassium influx through the Trk or other systems when the cells are cultivated at pH 7.5 (35). Furthermore, the DCCD-sensitive J_{K+} in the *focA* mutant, REK701, was decreased ∼40% compared with wild-type (Fig. 3 *A*). A similar decrease was measured in *focB* single and *focA-focB* double mutants (Fig. 3 *A*). However, the DCCD-sensitive J_{K+} decrease was not related to the F_OF_1 ATPase-dependent potassium flux. This might possibly suggest an interdependence between the Hyf-dependent FHL-2 complex and the potassium uptake systems (18). Alternatively, another potassium system, independent of Trk, might be active under these conditions (35). This would suggest a link between FocA-Trk-F_OF_1-ATPase, which is independent of the FocB channel.

Analysis of J_{K+} in formate-supplemented assays using wild-type cells revealed J_{K+} efflux with a rate of 0.14 mmol min^{-1} (Fig. 3 *A*). In all mutants, J_{K+} was residual or zero. This is an unexpected result, which might suggest that, in the presence of exogenous formate in the assays, the formate channels are involved not only in regulation of proton transport but also of potassium transport. In wild-type cells, J_{K+} was strongly dependent on F_OF_1-ATPase. Moreover, the DCCD-sensitive J_{K+} in the *focA* single mutant (REK701) and the *focA-focB* double mutant showed similar rates of potassium efflux as for the wild-type without DCCD treatment. This suggests that when FocA is absent, this disturbs further metabolism of exogenously added formate and its regulation in the cell.

In the glycerol assays using wild-type cells, J_{K+} reached 0.3 mmol min^{-1} (Fig. 3 *A*). In the *focA* mutant J_{K+} was 0.12 mmol min^{-1} and in the *focB* mutant it attained a value of 0.18 mmol min^{-1}. In the *focA-focB* double mutant it was ∼0. Upon addition of DCCD to wild-type cells, the direction of the potassium flux changed and potassium efflux was registered, reaching 0.8 mmol min^{-1} (Fig. 3 *B*). Similar changes were determined in the *focB* mutant, while in the mutants where there is no FocA present, DCCD had no effect.

When wild-type cells were grown under M2 conditions in glucose assays, the total J_{K+} was 0.18 mmol min^{-1} (Fig. 4 *A*). The flux was ∼20% lower compared with when the wild-type cells were grown under M1 conditions (compare Figs. 4 *A* and 3 *A*). This might be due to the changes in major bioenergetic properties of the cell, particularly the transmembrane proton gradient and membrane potential. Indeed, the F_OF_1-ATPase activity was significantly lowered under M2 conditions (see Fig. 6). In contrast to J_{K+}, the total J_{H+} was increased under this condition. It is known that TrkA-related potassium transport is dependent on F_OF_1-ATPase activity (3,18). This type of relationship has also been shown for *Mycobacterium smegmatis* where potassium has an essential role in pH homeostasis and in regulating the membrane potential (36). In the *focA* mutant, total J_{K+} was ∼0.1 mmol min^{-1}, which was ∼50% lower compared with that in wild-type cells. The data show that FocA affects potassium flux when exogenous formate is added to the growth medium, which was not the case for the cells grown under M1 conditions. In the *focB* mutant, the total J_{K+} was 0.24 mmol min^{-1}, which was increased by ∼30% compared with the wild-type (Fig. 4 *A*), while under M1 conditions a significant decrease of the total flux was registered (compare Fig. 3 *A*). In the double mutant total J_{K+} was 0.29 mmol min^{-1}, which was increased ∼60% compared with the wild-type.

Using formate as electron donor, potassium flux was negligible in wild-type and mutant cells grown under M2 conditions, but when DCCD was added to the assays using wild-type cells and cells of the *focA* mutant, potassium efflux was registered with a rate of 0.11 mmol min^{-1} (Fig. 4 *B*). In the *focB* single mutant and the *focA-focB* double mutant, addition of DCCD had no effect on potassium flux. A similar lack of change in potassium efflux was registered in the cells grown under M1 conditions. These data suggest that the presence of external formate in the medium changes the mechanism of formate channels, and this is particularly apparent for FocB. In glycerol assays in wild-type cells the potassium flux was residual and approximated zero (Fig. 4 *A*).

Total and F_OF_1 ATPase activity during mixed-carbon source fermentation

The total ATPase activity in wild-type cells grown under M1 conditions was determined to be ∼300 nmol P_i

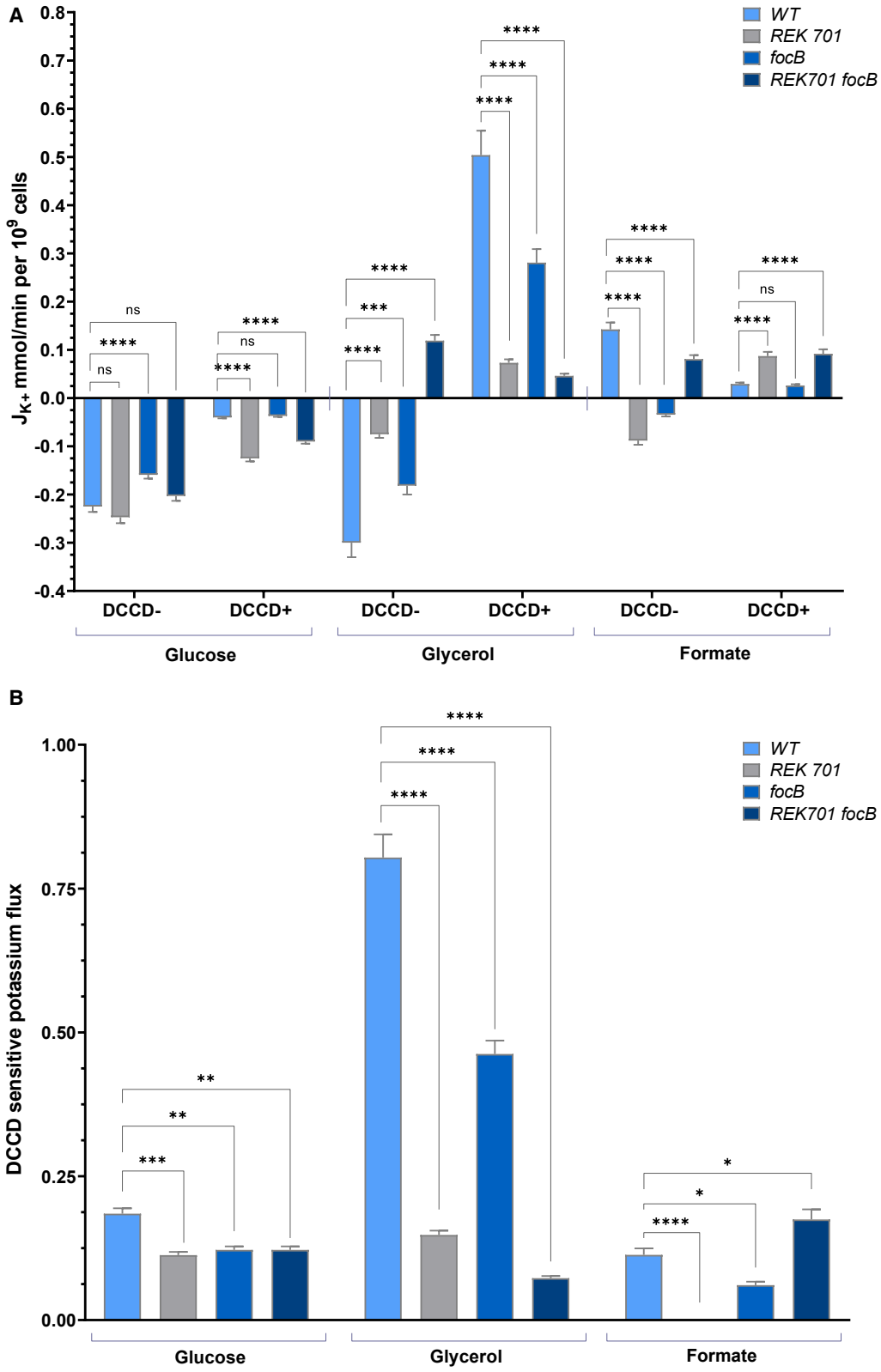

FIGURE 3 Total (*A*) and DCCD-sensitive (*B*) K^+ fluxes by whole cells of *E. coli* wild-type and *foc* mutants after growth until stationary phase in the presence of glucose and glycerol (M1 condition) at pH 7.5. In the assays glucose (2 g L^{-1}), glycerol (10 g L^{-1}), or formate (0.68 g L^{-1}) was added as energy source. DCCD (0.2 mM) was added to the assay medium where indicated.

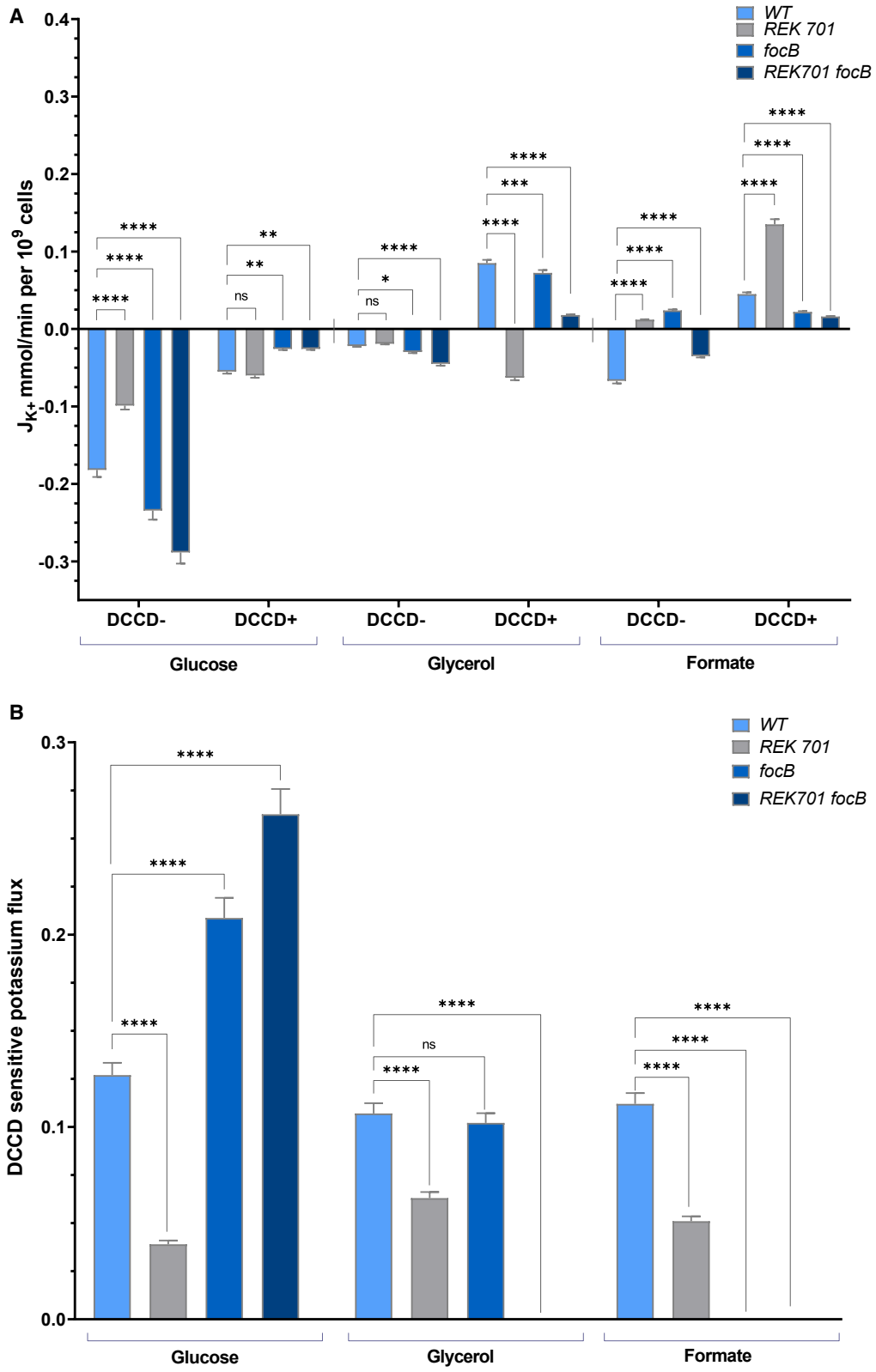

FIGURE 4 Total (*A*) and DCCD-sensitive (*B*) K^+ fluxes in whole cells of *E. coli* wild-type and *foc* mutants after growth until stationary phase in the presence of glucose, glycerol, and formate (M2 condition) at pH 7.5. In assays glucose (2 g L^{-1}), glycerol (10 g L^{-1}), or formate (0.68 g L^{-1}) was added as energy source. DCCD (0.2 mM) was added to the assay medium where indicated.

(min μg protein)$^{-1}$ (Fig. 5 *A*). In all *foc* mutants, except the *focA-focB* double mutant, the total ATPase activity was similar to that measured for wild-type cells. In the double mutant, the total ATPase activity was decreased by ~25% (Fig. 5 *A*). This decrease, when compared with the DCCD-treated cells (Fig. 5 *B*), was not due to the changes in the activity of F_OF_1-ATPase activity.

Generally, the addition of DCCD in the assays decreased the ATPase activity by ~90% (Fig. 5 *B*), which suggests that the total ATPase activity measured was mainly due to the activity of F_OF_1-ATPase (Fig. 5 *A*). Similar activities were detected for the mutants in the DCCD assays (Fig. 5 *B*). The addition of potassium ions reduced the DCCD-sensitive component of the ATPase activity by ~30%, while only in REK701 (*focA*) addition of potassium ions stimulated the DCCD-sensitive ATPase activity by ~35% (Fig. 5 *B*). DCCD-sensitive ATPase activity in the formate assays was not changed in wild-type or in the mutants, except in REK701 (*focA*) cells, where DCCD-sensitive ATPase activity was reduced by 30% compared with wild-type.

The wild-type cells grown under M2 conditions had similar total ATPase activity as in the wild-type cells grown under M1 conditions (Fig. 6 *A*); however, the DCCD-sensitive ATPase activity was reduced by ~35% in cells grown under M2 conditions (Fig. 6 *B*). This result suggests that external formate decreased the contribution of F_OF_1-ATPase activity compared with the cells grown under M1 conditions. Total and DCCD-sensitive ATPase activities in all mutants were similar to the activities measured for the wild-type (Fig. 6, *A* and *B*). However, in the REK701 (*focA*) strain, activity was increased by ~50% compared with the wild-type. Nevertheless, the contribution of F_OF_1-ATPase to the total ATPase activity was significantly lower, by ~60%, compared with the cells grown under M1 conditions where the F_OF_1-ATPase contribution was ~90% (Fig. 5). In REK701 (*focA*) the DCCD-sensitive ATPase activity was ~2.2-fold higher than wild-type, which means that the difference in the activity is due to the activity of the F_OF_1-ATPase (Fig. 6 *B*). The effect of potassium ions on DCCD-sensitive ATPase activity was determined only in the *focA-focB* double mutant, with a ~40% decrease observed when compared with wild-type (Fig. 6 *B*).

In the assays in which formate was added as electron donor, the DCCD-sensitive ATPase activity in wild-type cells was stimulated by ~60% (Fig. 6 *B*). In cells of the *focA* mutant, REK701, the addition of formate to the assays increased the DCCD-sensitive ATPase activity by ~1.6-fold, while in the *focA-focB* double mutant it had a similar value and was decreased approximately 2-fold compared with wild-type. Interestingly, in the presence of potassium and formate ions, the DCCD-sensitive ATPase activity in the wild-type remained unchanged, but in the *focB* mutant and in the REK701 (*focA*) mutant it was stimulated 60–70% (Fig. 6 *B*).

Transmembrane pH gradient

The pH gradient is one of the most important parameters that cells try to regulate to balance pH, ion gradients, and the general proton motive force. Many systems in the cell, including the secondary transport systems, contribute to the balancing of ion homeostasis (37), but this depends on various external parameters, such as pH, carbon source availability and their respective concentrations (38). When wild-type cells were grown under M1 conditions, the transmembrane pH gradient (ΔpH) of the cells was equal to 1.13 units (Fig. 7). The REK701 (*focA*) mutant had a ΔpH of 0.95 units, which is ~0.2 units less than that measured for the wild-type or the other mutants. Surprisingly, the ΔpH in the *focA-focB* double mutant was also similar to that of the wild-type, which suggests that when both formate channels are absent, or inactive, the cells regulate proton cycling differently and, again, this could be related to slower equilibration of formic acid across the membrane.

When wild-type cells were grown under M2 conditions, the ΔpH was 0.88 units, which is lower by 0.25 units compared with when cells were grown on glucose and glycerol (Fig. 7). In the *focB* mutant, the ΔpH value was also similar to the wild-type strain. Notably, however, in the *focB* mutant the cytoplasmic pH was determined to be 7.5 units, while in the wild-type and the other mutants analyzed, the intracellular pH was 7.25 units (data not shown). This suggests that the cells try to balance the internal pH by increasing the DCCD-sensitive F_OF_1-ATPase activity by transporting protons out of the cell. In the *focA* mutant and the *focA-focB* double mutant, the ΔpH values were 0.5 units (Fig. 7). The decrease in the ΔpH by ~0.38 units compared with the wild-type is significant and suggests different regulation of pH homeostasis when exogenous formate is present. In particular, when compared with the conditions where there is no added formate, only the REK701 (*focA*) strain showed a decrease of ΔpH, while the data obtained under M2 conditions, where exogenous formate is present during growth, strongly suggests that FocA is important in balancing pH in stationary phase cells.

DISCUSSION

In this study we examined the effect of exogenously added formate on key bioenergetic parameters in

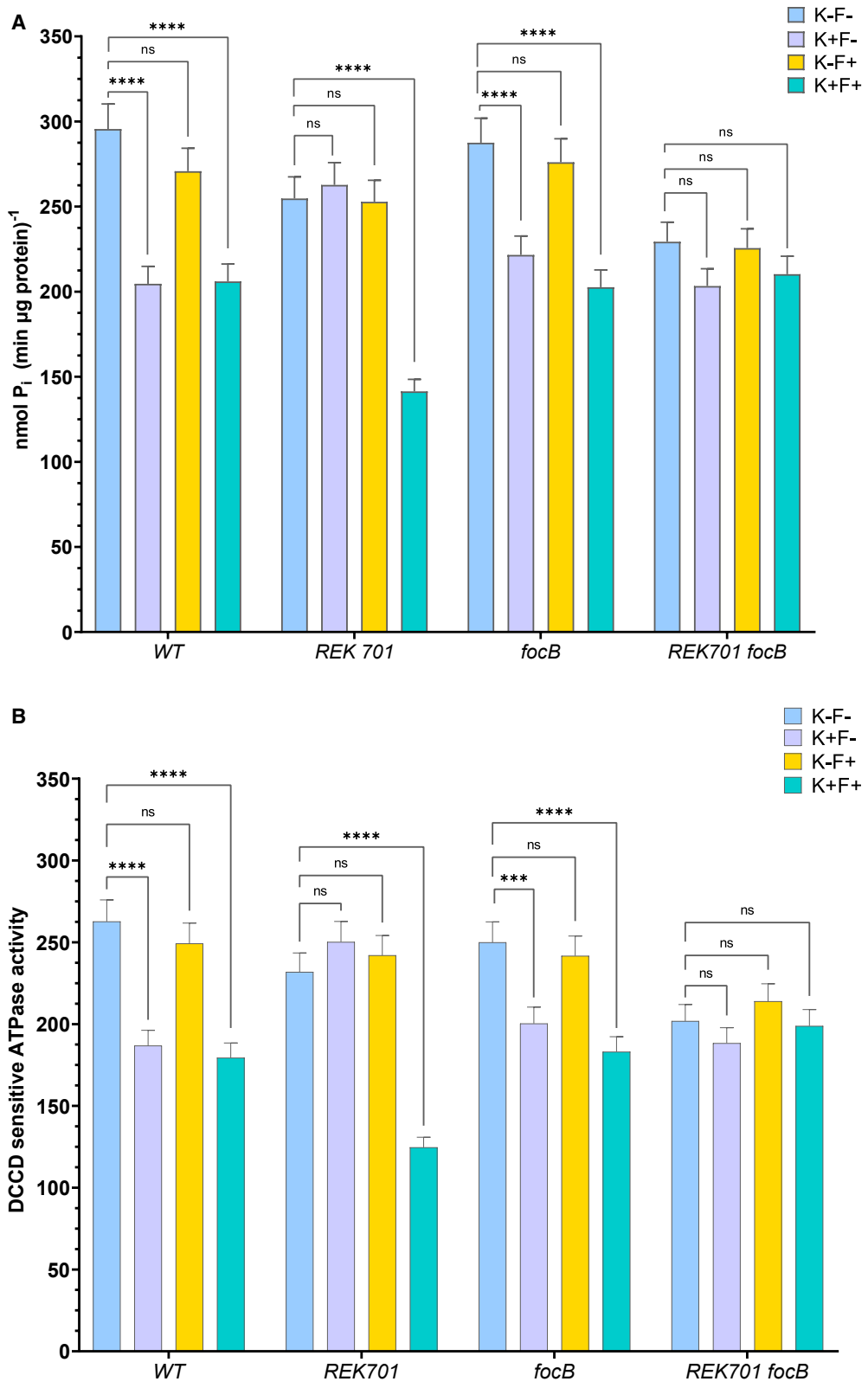

FIGURE 5 Total (*A*) and DCCD-sensitive (*B*) ATPase activity of membrane vesicles derived from cells of *E. coli* wild-type and *foc* mutant strains grown fermentatively at pH 7.5 to stationary phase in the presence of glucose and glycerol (M1 condition). ATPase activity was calculated as the difference between the values determined in assays in which no DCCD was added or DCCD (0.1 mM) was added into the assay medium. Potassium ions (100 mM) or formate (10 mM) was added in the assays and is indicated as K^+ or F^+.

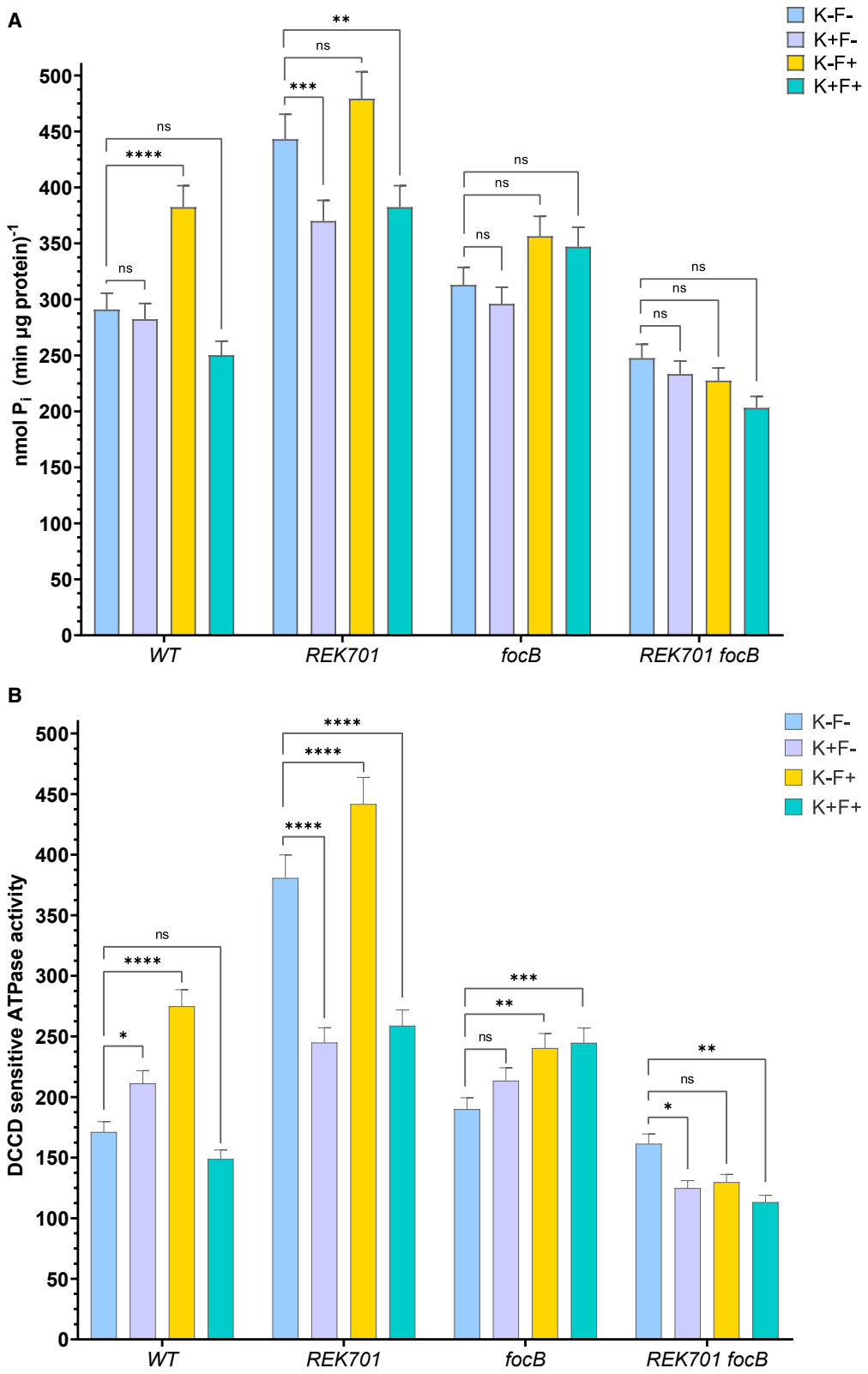

FIGURE 6 Total (*A*) and DCCD-sensitive (*B*) ATPase activity of membrane vesicles derived from cells of *E. coli* wild-type and *foc* mutant strains grown fermentatively at pH 7.5 to stationary phase in the presence of glucose, glycerol, and formate (M2 condition). For others see legend to Fig. 5.

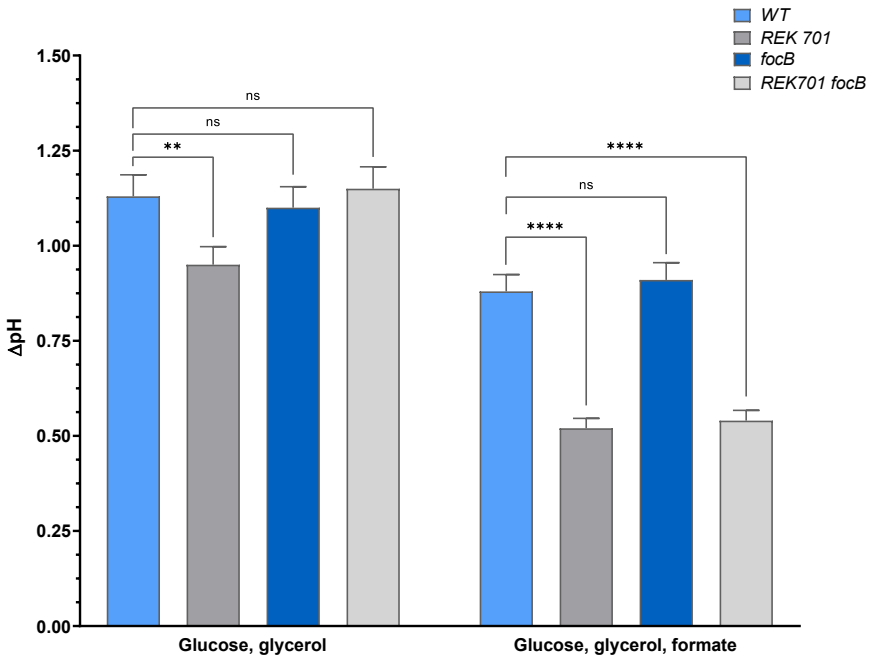

FIGURE 7 Values of ΔpH determined for *E. coli* wild-type and *foc* mutant strains grown fermentatively at pH 7.5 with glucose and glycerol (M1 condition) or with glucose, glycerol, and formate (M2 condition). ΔpH was calculated as the difference between pH_{in} and pH_{ex}. For other see materials and methods.

mutants lacking one or both of the formate channels, FocA and FocB. The cells were grown on glucose and glycerol at pH 7.5, with or without formate addition, and cultivated until they entered the stationary phase. Before harvesting of the cells, the extracellular formate concentration in the medium was determined. It was shown that, when cells were grown on glucose and glycerol (M1 condition), in wild-type cells and mutants the formate concentration was similar for all strains and in the range of 14–15 mM (see Fig. S2). When cells were grown in glucose and glycerol conditions plus exogenously added formate (M2 condition), in wild-type and single mutants the formate concentration was in the range of 16–17 mM, while for the culture medium used for growth of the *focA-focB* double mutant the formate concentration was 21 mM. This suggests that the absence of both formate channels disturbs the influx of formate into the cells. Under these conditions in stationary phase cells, the FHL-1 complex is induced and active (1,39) and, therefore, these data demonstrate that the cells were all in roughly the same metabolic state for the subsequent analyses.

In wild-type cells, the differences in proton or potassium fluxes in response to addition of different electron donors (glucose, glycerol, or formate) can be explained by the differences in their respective metabolism. In particular, during glucose utilization greater acidification of the medium occurs because of the different acids produced, while during glycerol utilization mainly potassium flux was increased. Exogenous formate uptake and proton flux is significantly reduced, as formic acid is mainly neutralized by the cells via its disproportionation by the FHL-1 complex producing H_2 and CO_2. The residual formate not metabolized by FHL-1 is translocated out of the cells along with a proton, mainly by FocA, resulting in acidification of the medium. The presence of formate during growth (M2 conditions) caused a significant increase in the proton flux when glucose was added as electron donor, presumably due to enhanced formic acid efflux (1,40,41), while using glycerol and formate as electron donor resulted in only residual proton fluxes being measured. Similar results were obtained for potassium fluxes. These results indicate that glucose metabolism results in increased proton efflux from the cells.

Notably, exogenously added formate changed the contribution of the F_OF_1-ATPase in balancing the total proton and potassium fluxes in the cells and this was most noticeable when the gene encoding either of the formate channels was deleted. Exogenous formate as electron donor increased the DCCD-sensitive proton flux, especially in the *focA* mutant, which indicates that external formate, or more likely formic acid, is imported into the cells and is disproportionated to H_2 and CO_2 by the FHL complex and suggests that the increased activity of the F_OF_1-ATPase compensates for the lack of FocA to help balance intracellular pH (9). This also agrees with an earlier demonstration that, in cells of a *focA* mutant grown at pH 7.5, the rate of H_2 production is significantly increased in the cells grown under the conditions where exogenous formate is added (9). This also correlates with recent

findings showing that exogenous formate is directly converted to H_2 and CO_2, while the internal formate produced from glucose is translocated out of the cell (14,42). The possibility that the F_OF_1-ATPase is linked to this process is supported by the data in this report, especially where an increase in F_OF_1-ATPase activity of ~70% was observed upon formate addition in assays. Importantly, with the exception of the *focA* mutant, the DCCD-sensitive ATPase activity was lowered by ~30–40% in the cells cultivated in the presence of exogenous formate, which strongly suggests involvement of FocA in this process. These data suggest that, directly or indirectly, F_OF_1-ATPase activity is involved in monitoring the presence of exogenous formate (see Fig. 8).

The results for DCCD-sensitive potassium fluxes in cells grown on glucose and glycerol show that the decrease in the flux of potassium in a *focB* mutant is not due to the activity of the F_OF_1-ATPase, which suggests that FocB might influence potassium flux via TrkA, or possibly alternative systems (43). It is known that the *focB* gene is located at the end of the *hyf* operon encoding FHL-2 (10), which has been shown previously to influence potassium flux (17) and likely is functional in late stationary phase cells.

When exogenous formate was present during growth, the total potassium flux was decreased in a *focA* mutant, but not in a *focB* mutant, and this decrease was F_OF_1-ATPase dependent. Moreover, the potassium flux data in the *focB* mutant grown in the presence of formate underlines the importance of F_OF_1-ATPase activity in balancing ion fluxes when the formate channels are absent; this effect was not observed when the *focB* mutant was grown on glucose plus glycerol alone. These data suggest that the cells use the energy from ATP hydrolysis by the F_OF_1-ATPase to balance internal and external pH when formate is present in the medium and in the absence of the formate channels. While the disturbance of both channels increased the potassium flux by ~60% compared with wild-type, the effect of a *focB* mutation (but $FocA^+$) was lower by ~30%. The proposal of an involvement of these channels in helping balance intracellular pH was also supported by the lowered ΔpH (by 0.3 pH units) of the mutants compared with wild-type.

A recent proposal has also suggested that the FHL-1 and -2 complexes and the formate channels contribute to pH homeostasis of the cell during fermentation (33). Moreover, in vitro studies have shown that the FocA channel reconstituted in artificial membranes has different translocation ability depending on membrane potential and ion concentration (44). Moreover, in a *fhlA* mutant that cannot synthesize the FHL-1 complex, and thus formate cannot be disproportionated to H_2 and CO_2, an increased potassium flux is observed during the fermentation of glucose, glycerol, and formate (41). The higher potassium flux into the cells could be due to the increased membrane potential, or to changes in osmotic pressure, which also needs to be regulated.

It is well established that the first response of the cell to the changes in osmotic pressure is via regulation of the potassium flux (34). Moreover, in the *focA-focB* double mutant, the DCCD-sensitive proton/potassium ratio was approximately 4, while in the wild-type the ratio was 2. In contrast, in the *focA* mutant, the DCCD-sensitive ratio of proton/potassium flux was dissipated and equivalent to 23, which was mainly due to the lack of DCCD-sensitive potassium flux (Table 2). It appears that the cells try to modulate

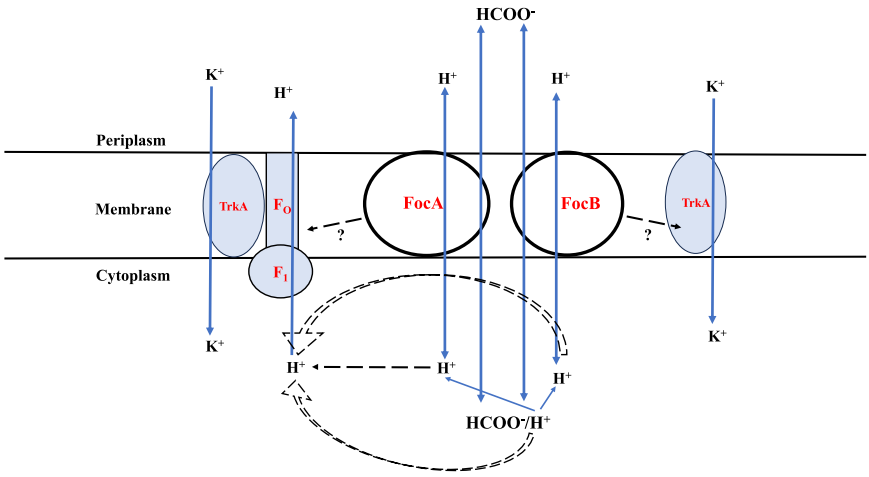

FIGURE 8 For a Figure360 author presentation of this figure, see https://doi.org/10.1016/j.bpr.2025.100225. Figure360▷

Schematic representation of the working mechanism of FocA- and FocB-dependent proton and potassium fluxes. Dashed arrows represent the possible compensatory role of proton efflux by F_OF_1-ATPase to balance pH. In the absence of exogenously supplied formate, FocB is suggested to influence TrkA-dependent potassium transport, or other similar systems, while in the presence of formate FocA has the same influence on TrkA. Exogenous formate impacts the F_OF_1-ATPase activity to increase its contribution to balancing proton or potassium fluxes. The question marks suggest possible physical interactions. In the interest of clarity, the FHL-1 complex, which converts formic acid to H_2 and CO_2, is not shown.

TABLE 2 Ratio of DCCD-sensitive proton/potassium fluxes in *E. coli* during mixed-carbon fermentation at pH 7.5 when glucose is the electron donor

	pH 7.5	
	Gluc+glyc (M1 condition)	Glu+glyc+form (M2 condition)
Wild-type[a]	1.44 ± 0.1	2.04 ± 0.1
JW2477	7.45 ± 0.2	5.32 ± 0.2
REK701	4.26 ± 0.2	4.61 ± 0.1
REK701ΔfocB	5.44 ± 0.1	23.07 ± 0.3

[a]Both wild-types (BW25113 and MC4100) used showed similar results.

the DCCD-sensitive proton/potassium ratio in an attempt to maintain pH homeostasis by producing H_2 and CO_2 by the FHL-1 complex, as has been recently proposed (40). Alternatively, cells also might attempt to balance pH by increasing the proton flux via the F_OF_1-ATPase. Thus, the involvement and contribution of ATP hydrolysis by the F_OF_1-ATPase likely increases when exogenous formate must be removed by FocA-FHL-1-dependent uptake and disproportionation (1,33).

It has been shown earlier that wild-type cells try to regulate the formate concentration outside the range of ∼10 mM, but when there is a lack of "formate-neutralizing" FHL-1 pathway, the cells accumulate formate outside the cells in the range of 30–40 mM (13,41). In wild-type cells, when they produce formate and translocate it to the external medium, then it is reimported for disproportionation by FHL-1. However, when exogenous formate is present from the beginning of growth, formate-dependent synthesis of the FHL-1 complex must be initiated earlier (39), which requires earlier pH homeostatic mechanisms to aid this process and these include the F_OF_1-ATPase (16). It seems that cells somehow distinguish between internally generated and exogenously supplied formate (14) and thus import of exogenous formate into the cells appears to be directly coupled to the activity of the FHL complex (14,42), while internally generated formate can be coupled to cotransport with protons out of the cell (1). It has been shown that the transport of other fermentation products such as succinate (45) and lactate (46) are coupled to proton symport and aid in generation of a membrane potential. It is proposed that transport of formic acid has a similar function in stationary phase cells. When formate channels are absent and formate instead of being transported out is further converted to H_2 and CO_2, one proton is used to generate hydrogen, while the rest can be effluxed via the F_OF_1-ATPase-dependent mechanism and thus F_OF_1-ATPase may be able to compensate for the loss of the ability of the cell to transport protons via a symport mechanism with formate via FocA/B channels (see Fig. 8).

The TELP theory, proposed for mitochondria (6), might also aid in understanding the results obtained in our study. During fermentation, H^+ are generated as a result of the dissociation of organic acid products (e.g., formate) and F_OF_1-ATPase has been suggested as the main pump for the efflux of these H^+ from the cytoplasm, driven by ATP hydrolysis. Under our experimental conditions, when bacteria ferment the mixture of carbon sources, the formate channels could transport formate anion/proton ions in or out using a symport mechanism with a 1:1 ratio. In the absence of the FocA or B channels, this is compensated by an increase in the DCCD-sensitive transport of H^+ via F_OF_1-ATPase. As suggested, H^+ might be transferred from Foc channels to F_OF_1-ATPase for efficient energy transduction. A simplified schematic working model summarizing these findings for the Foc channels, potassium systems, and the F_OF_1-ATPase is presented in Fig. 8.

Overall, the data show that, during growth at pH 7.5, and when exogenous formate is available, the FocA and FocB channels play an important role in fermentatively growing and in stationary phase cells. It is suggested that they control formate translocation and couple this with efflux of protons using a symport mechanism (1). Excessive formate levels direct formate and protons to be neutralized by the disproportionation of formic acid through the activity of the FHL-1 complex, or the protons can be translocated out of the cell by the F_OF_1-ATPase to maintain pH homeostasis. It is conceivable that when exogenous levels of formate are high, FocA is primarily responsible for balancing pH through proton transport or cycling, while FocB's role is mainly balancing the potassium ion gradient in stationary phase cells.

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AUTHOR CONTRIBUTIONS

L.G. and A.B. carried out the experiments. A.V., A.P., G.S., and K.T. designed the experiments and analyzed the data, drafted the manuscript, and conceived the study. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

SUPPORTING MATERIAL

Supplemental information can be found online at https://doi.org/10.1016/j.bpr.2025.100225.

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