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Interplay between the Conserved Pore Residues Thr-91 and His-209 Controls Formate Translocation through the FocA Channel

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Keywords
Formate translocation · Anion channel · FocA pore · Threonine-91 · Histidine-209

Abstract
The formate channel A (FocA) belongs to the formate-nitrite transporter (FNT) family, members of which permeate small monovalent anions. FocA from *Escherichia coli* translocates formate/formic acid bi-directionally across the cytoplasmic membrane during fermentative growth. Two residues are particularly well-conserved within the translocation pores of FNTs: threonine-91 and histidine-209, based on *E. coli* FocA numbering. These residues are located at the tips of two broken transmembrane helices and control anion passage. H209 is the only charged residue within the pore and interacts with T91. Here, we addressed the role of the T91-H209 interaction network in the permeation of formate in vivo through FocA by performing an extensive amino acid-exchange study. Monitoring changes in intracellular formate using a formate-responsive *fdhF*$_P$*::lacZ* reporter system revealed that T91 is essential for the ability of FocA to translocate formate bi-directionally. Only exchange for serine was partially tolerated, indicating that the hydroxyl group of T91 is mechanistically important. Substitution of H209 with N or Q was previously shown to convert FocA into a formate efflux channel. We show here that residue exchanges A, I, and T at this position resulted in a similar phenotype. Moreover, efflux function was confirmed for these FocA variants by measuring excreted formate in the culture medium. Substitution of bulky or charged residues for H209 prevented bi-directional formate passage. Studies using hypophosphite, a toxic analogue of formate taken up by FocA, and which causes impaired growth, confirmed that T91 and H209 substitutions essentially abolished, or drastically reduced, FocA's translocation activity, as shown by effects on growth rate. The exceptions were T91S- and T91Y-exchange variants that retained partial ability to take up inhibitory hypophosphite. Together, our findings indicate that T91 is essential for formate permeation in both directions; however, it is particularly important to allow anion efflux. Moreover, H209 is essential for formate uptake by FocA, strongly suggesting that protonation-deprotonation of this residue plays a role in formate uptake. Finally, our results substantiate the premise that efflux and influx of formate by FocA are mechanistically distinct processes that are controlled by the interplay between T91 and H209.

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Introduction

During mixed-acid fermentation *Escherichia coli* and other enterobacteria non-oxidatively cleave pyruvate to formate and acetyl-CoA using the glycyl-radical enzyme pyruvate formate-lyase (PflB) [Knappe and Sawers, 1990]. The bulk of the formate is translocated out of the cytoplasm to the periplasm by formate channel A (FocA) [Suppmann and Sawers, 1994; Kammel et al., 2021, 2022], which is the archetype of the formate-nitrite transporter (FNT) family of anion channels. FNT channels are found widely distributed among anaerobic microorganisms and each translocates a specific monovalent anion, usually either into or out of the cell [Wu et al., 2015; Mukherjee et al., 2017; Zeng et al., 2021]. FocA appears to be unusual in that it is able to translocate formate/formic acid in both directions, depending on the pH [Suppmann and Sawers, 1994; Beyer et al., 2013]; it is currently unresolved whether the acid or the conjugate base is translocated, and thus for convenience henceforth we will refer simply to formate, except where formic acid is specifically meant. At neutral pH formate is mainly translocated from the cytoplasm into the periplasm. But when the pH of the growth medium decreases substantially below 7, due to the accumulation of organic acids such as acetic acid, lactic acid, succinic acid, and mainly formic acid, the latter is taken back up into the cytoplasm. The reimport of formic acid would partially offset the acidification of the growth medium because once inside the cell its accumulation induces synthesis of the formate hydrogenlyase complex, which disproportionates it into dihydrogen and carbon dioxide [Sargent, 2016]. How FocA is able to control the direction of formate translocation is not completely understood, and is the focus of this study.

FNT channels are all homopentamers and they share a structural fold with the aqua- and glyceroporin family of channels [Sui et al., 2001; Lü et al., 2013; Waight et al., 2013]. Each protomer of the pentamer has a hydrophobic pore that has two constriction sites, which restrict anion access [Wang et al., 2009; Waight et al., 2010; Lü et al., 2011]. The pore is formed from two transmembrane alpha-helices (TM2 and TM5) that are each disrupted to form two shorter alpha-helices joined by a loop. The two loop-like structures are termed the Ω- and S-loops and are folded such that the tips of each converge from above and below the plane of the membrane, respectively [Wang et al., 2009] (Fig. 1a). The upper S-loop, located close to the periplasmic side of the membrane, is held rigidly in position, while the lower, converging Ω-loop, at the cytoplasmic side of the membrane, appears to be more flexi-

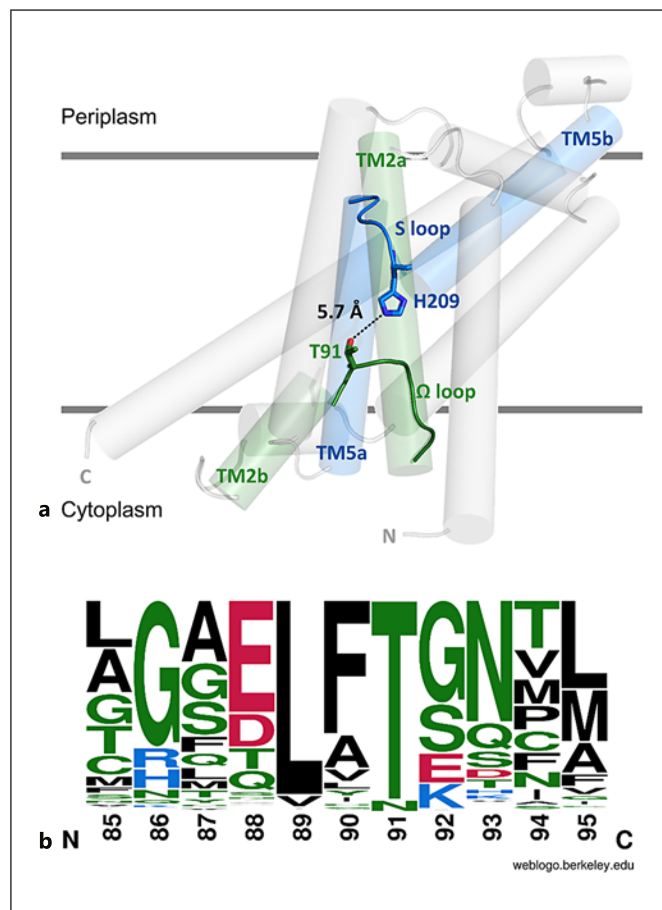

Fig. 1. Conservation of threonine residue 91 within the pore of FNT channels and interaction with histidine residue 209. **a** Depiction of the locations of T91 and H209 within the FocA pore (PDB structure 3KCU [Wang et al., 2009]). One of the protomers was visualized in cartoon representation with transmembrane helices depicted as cylinders. The residues T91 (green) and H209 (blue) are displayed in their stick representation and the adjoining transmembrane helices 2 and 5 (TM2/5 a and b) and both the Ω- and S-loops are highlighted in light green and light blue, respectively. The distance between H209's *pros*-N hydrogen and the hydroxyl-group of T91 was determined using PyMOL (The PyMOL Molecular Graphics System, version 2.5, Schrödinger, LLC). **b** Conservation plot comparing the amino acid residues between positions 85 and 95 using 258 FNT channels, which were used to create a WebLogo 3 stacking plot ([Crooks et al., 2004]; see the Materials and Methods section). The residues are aligned with native FocA from *E. coli*. Amino acid residues displayed in black have an apolar side chain (A, F, I, L, M, P, V, W), while polar side chains are indicated in green (C, G, N, Q, S, T, Y). Acidic amino acids are shown in red (D, E) and basic amino acid residues are shown in blue (H, K, R). A similar stacking plot analysis for H209 has been presented previously [Kammel et al., 2022].

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ble, and in FocA can adopt different positions within the pore. This suggests that the position of the Ω-loop might control anion access, i.e., through "opening" and "closing" of the pore [Lü et al., 2013; Waight et al., 2013]. The tip of each loop has a conserved amino acid residue, i.e., found in most FNT family members, suggesting a common mechanism governs all of these channels, irrespective of the permeating anion. The tip of the S-loop has a histidine residue (H209 in FocA of *E. coli*) and this represents the only charged amino acid residue within the core of the pore (Fig. 1a). The tip of the Ω-loop has a threonine residue (T91). Because H209 can be protonated ($pK_a = 6$), it has been proposed that protonation might be involved in the anion uptake mechanism and could explain the pH dependence of the inward permeation of formate through FocA [Suppmann and Sawers, 1994; Sawers, 2005]. Evidence in support of this proposal has been obtained recently, whereby exchange of the conserved H209 residue in FocA of *E. coli* for an asparagine or glutamine residue resulted in FocA functioning only in the efflux direction [Kammel et al., 2022]. Notably, there are a few examples of FNT channels with either N or Q at the tip of the S-loop, suggesting that they might be uni-directional channels for a particular anion.

Like H209 [Kammel et al., 2022], T91 is highly conserved within FNT channels (Fig. 1b). The functional role of T91 on the tip of the Ω-loop with respect to formate translocation by FocA is, however, unclear. Structural studies have shown that T91 can form a hydrogen bond with H209 [Waight et al., 2010; Lü et al., 2011, 2012], while the structures of other pores reveal it is separated from H209 by more than 5 Å. These observations have led to the suggestion that the position of T91 on the Ω-loop, and its proximity to, or interaction with, H209, might govern whether the pore is open or closed [Lü et al., 2013; Waight et al., 2013]. Notably, T91 also coordinates with a water molecule in the pore and this has led to the suggestion that the interplay among H209, T91, and the bound H_2O possibly influences the directionality of formate translocation [Lü et al., 2012]. Moreover, because uptake of the formate anion via a "channel" into the negatively charged cytoplasm would be thermodynamically challenging, coupled with the fact that the core of the pore is hydrophobic, this has led to the suggestion that uncharged formic acid is translocated into the cytoplasm [Wiechert and Beitz, 2017; Helmstetter et al., 2019]. However, this would reduce the proton gradient due to the symport of an H^+ with each formate translocated. Consequently, in an alternative hypothesis, the H209-T91-H_2O network has been proposed to recapture the proton during the passage of the undissociated acid [Lü et al., 2012], which would be more advantageous, in terms of energy conservation, for a fermenting bacterium.

In a previous mutagenesis study, we provided preliminary evidence in support of an important function for T91 in the translocation of formate by FocA [Hunger et al., 2014]. In the current study, we have carried out a more extensive analysis of the roles of T91 and H209 in formate translocation by *E. coli* FocA. Our findings demonstrate that the interplay between these two amino acid residues is crucial in governing the directional permeation of formate through FocA.

Results

Exchanging H209 for Either Ala, Asn, Gln, Ile, or Thr Converts FocA into an Efflux Channel for Formate

When either of the amide amino acid residues N or Q is introduced in place of histidine at amino acid position 209 in the core of the FocA pore, *E. coli* cells synthesizing these FocA variants very efficiently excrete formate during fermentation [Kammel et al., 2022]. To determine whether any other amino acid residue exchange at position H209 resulted in a similar FocA phenotype, we introduced codons into *focA* that decode as A, D, F, I, K, T, or W in place of H. The genes encoding these variants were introduced on a plasmid into the *focA* mutant DH701 (see online suppl. Tables S1 and S2 supplementary information; for all online suppl. material, see www.karger.com/doi/10.1159/000524454) and, after anaerobic growth, changes in formate-responsive $fdhF_p$::*lacZ* reporter expression within the cell were monitored. Thus, changes in levels of enzyme activity directly reflect relative changes in formate levels within the cell [Beyer et al., 2013]. In comparison to the β-galactosidase enzyme activity in DH701 synthesizing native FocA, cells synthesizing either the alanine, the isoleucine, or the threonine variant ($FocA_{H209A}$, $FocA_{H209I}$, or $FocA_{H209T}$, respectively), showed barely detectable levels of β-galactosidase enzyme activity, and thus had essentially no formate in the cells (Fig. 2a). Due to the fact that intracellular formate must accumulate to a low mM concentration in order to induce expression of the *fdhF* gene promoter [Hopper and Böck, 1995; Kammel et al., 2022], this result suggests that the intracellular formate concentration was below the threshold required to induce *fdhF* expression. This could be because formate was not made, or, more likely, because it was immediately translocated out of these cells by these FocA variants [Kammel et al., 2022]. To distin-

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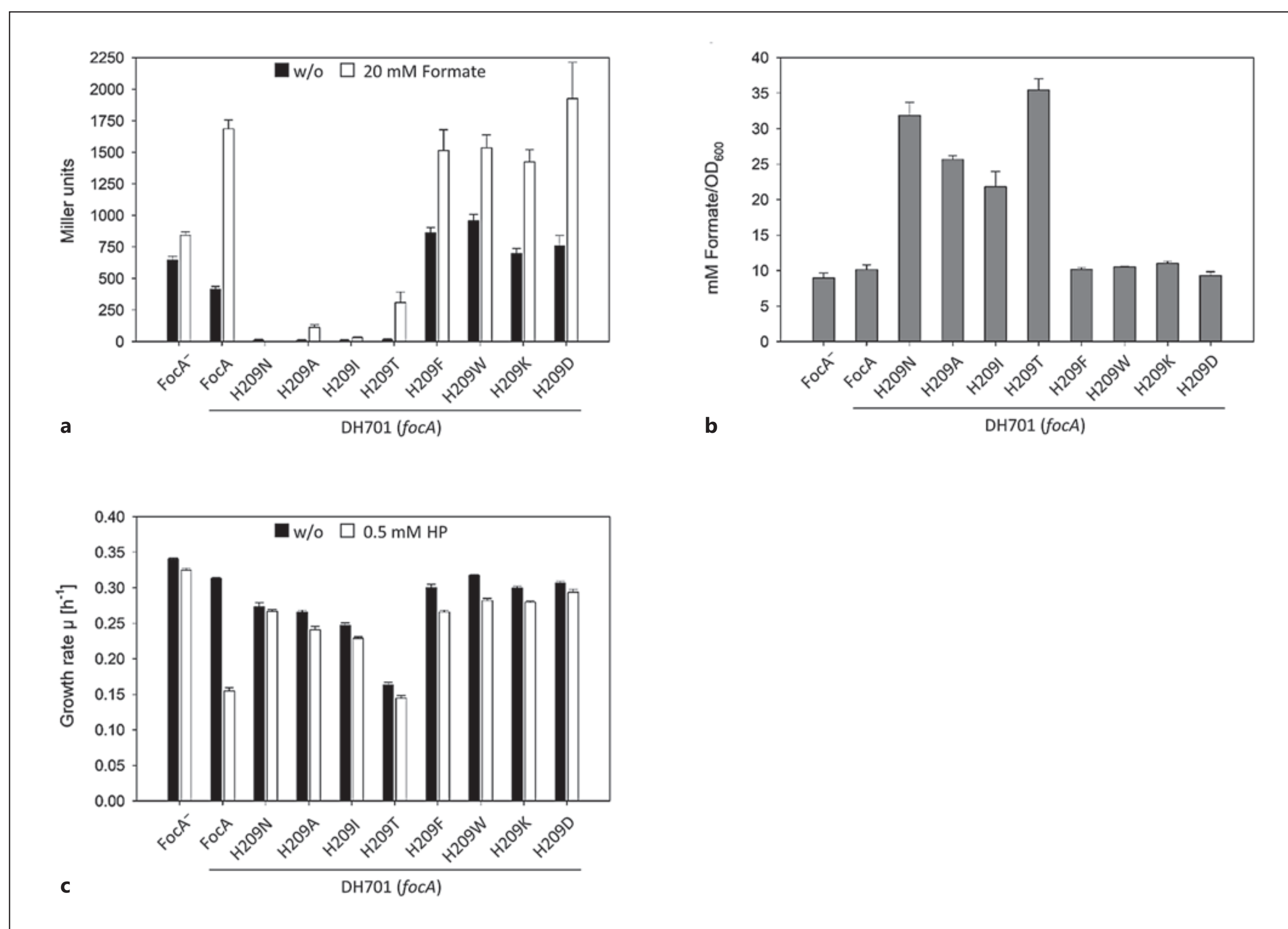

Fig. 2. Histidine-209 is essential for bi-directional formate translocation via FocA. Parameters determined to monitor formate translocation were investigated in the *focA* mutant DH701 (*focA*) and in DH701 transformed with plasmids carrying a *focA* gene encoding the indicated H209 variants. The negative control is labelled FocA⁻ and indicates the strain without FocA, while the positive control is labelled FocA, and indicates re-introduction of the parental FocA channel. Strains were grown anaerobically in glucose-M9-minimal medium (see the Materials and Methods section). **a** Formate-induced β-galactosidase enzyme activity of the respective strains was determined in cells grown to late-exponential phase without (black histogram) and with exogenously added 20-mM sodium formate (white histogram). Please see Kammel et al. [2022] for comparable values for DH4100 (wild type). **b** The concentration of formate in the culture medium after growth of the same strains shown in panel **a** was measured using HPLC analysis. **c** The sensitivity of the respective strains towards sodium hypophosphite was analysed by determination of anaerobic growth rates in the absence (black histograms) and in the presence of 0.5-mM sodium hypophosphite (white histograms). All experiments were performed with minimally three biological replicates, with each assay performed in duplicate.

guish between these alternatives, formate levels in the culture medium were determined (Fig. 2b). When DH701 synthesized either $FocA_{H209A}$, $FocA_{H209I}$, or $FocA_{H209T}$ ~20–35-mM formate OD_{600}^{-1} was measured in the growth medium, while the same strain synthesizing native FocA excreted less than 50% of this concentration (10.1 ± 0.7-mM formate OD_{600}^{-1}). As a control, the experiment was also performed with a culture of DH701 synthesizing $FocA_{H209N}$, which, as previously reported [Kammel et al., 2022], showed a phenotype similar to DH701 synthesizing $FocA_{H209A}$, $FocA_{H209I}$, or $FocA_{H209T}$ (Fig. 2a, b).

Finally, it has been reported that when DH701 synthesizes the $FocA_{H209N}$ variant, cells have a considerably reduced growth rate under fermentative conditions, which was suggested to be through the loss of a source of CO_2 for carboxylation reactions, due to formate efflux [Kam-

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mel et al., 2022]. Analysis of the anaerobic growth rates for DH701 synthesizing either $FocA_{H209A}$, $FocA_{H209I}$, or $FocA_{H209T}$ revealed these strains showed a similar reduction in growth rate to that observed for DH701 synthesizing $FocA_{H209N}$ (Fig. 2c). Notably, DH701 synthesizing $FocA_{H209T}$ showed the most severe growth reduction (almost 50% reduction in growth compared to DH701 synthesizing native FocA). Taken together, these results clearly indicate that when DH701 synthesizes FocA variants in which H209 is exchanged for an amino acid residue with either a small polar or non-polar side chain, essentially no expression of formate-dependent *fdhF$_p$::lacZ* occurs because of efficient formate efflux from the cytoplasm.

The side chains of alanine and isoleucine cannot form H-bonds, and this precludes an interaction with T91 (Fig. 1a). In contrast, the side chain of threonine is a potential hydrogen bond acceptor or donor. Therefore, to determine the impact that an amino acid residue with either a large (phenylalanine or tryptophan) or charged (lysine or aspartate) side chain has on formate translocation by FocA, the *focA* gene on plasmid pfocA was mutated at codon 209 to deliver the correspondingly altered *focA* genes (see online suppl. Table S2 supplementary data). The FocA variants constructed included $FocA_{H209F}$, $FocA_{H209W}$, $FocA_{H209K}$, and $FocA_{H209D}$, and their cognate plasmids (online suppl. Table S1) were introduced into strain DH701 (*focA*) and *fdhF$_p$::lacZ* expression after fermentative growth with glucose was determined (Fig. 2a). All four of these FocA variants had higher β-galactosidase enzyme activity compared to that of the *focA* mutant DH701, which indicates that these residue exchanges led to inactive, or poorly functional, FocA proteins in the efflux direction. Formate levels measured in the culture medium were similar to those determined for DH701 (Fig. 2b). Western blot analysis of selected examples of these variants demonstrated that they were stably inserted into the membrane of strain DH701 (online suppl. Fig. S1), thus obviating lack of synthesis as being the cause of the efflux-negative phenotype. Together, these results suggest that the exchange of H209 for a sterically larger aromatic amino acid residue, or for an amino acid residue with a charged side chain, impedes effective formate translocation across the cytoplasmic membrane.

Uptake of Exogenous Formate Is Dependent on H209 in the Pore of FocA

Next, we supplemented the anaerobic growth medium with 20-mM sodium formate and measured the effect on *fdhF$_p$::lacZ* expression [Beyer et al., 2013; Kammel et al., 2021, 2022]. Strain DH701 lacking FocA showed only a minor increase in formate levels (change in β-galactosidase enzyme activity) of 30% upon exogenous formate supplementation, when compared with no formate addition (Fig. 2a). When strain DH701 synthesized the native FocA protein, encoded from plasmid pfocA, a 2-fold increase in intracellular formate level, compared to that of a strain producing no FocA, was measured (Fig. 2a); thus, any increase in *lacZ* expression above 30% was due to formate uptake via FocA. DH701 synthesizing either of the $FocA_{H209A}$, $FocA_{H209I}$, or $FocA_{H209N}$ variants showed either no, or a minimal, formate uptake when exogenous formate was added to the culture (Fig. 2a). This reveals that these variants do not take up formate efficiently. Surprisingly, cells synthesizing the $FocA_{H209T}$ variant showed an approximately 25-fold difference in intracellular formate level (reflected as increased β-galactosidase enzyme activity) between an experiment without added formate or with 20-mM formate added to the growth medium (Fig. 2a). This suggests that the $FocA_{H209T}$ variant is at least partially able to permeate formate in the uptake direction. It should be noted, however, that the regulation pattern, i.e., formate level, in cells synthesizing this variant was similar to that of cells synthesizing native FocA, just overall significantly lower (~5-fold) (see Fig. 2a).

The F, W, and K variants all showed a similar 1.6- to 2-fold increase in activity (reflecting the intracellular formate level) compared to when no formate was added, while cells synthesizing the aspartate variant had an even higher formate level (~2.6-fold) (Fig. 2a). Generally, these H209 FocA variants (substitutions with either F, W, K, or D) showed comparable levels of intracellular formate like native FocA, when challenged with 20-mM exogenous formate (Fig. 2a). These data suggest that a capability for exogenous formate uptake was retained by five of the variants. We can exclude that this effect was exclusively due to passive diffusion of formic acid into the cells because, as mentioned above, the *focA* mutant DH701 failed to show a similar increase in *fdhF$_p$::lacZ* expression.

The Hypophosphite-Sensitivity Phenotype of Anaerobic E. coli Is Dependent on H209 in the Pore of FocA

The formate analogue sodium hypophosphite irreversibly inhibits PflB [Plaga et al., 1988], resulting in reduced anaerobic growth [Suppmann and Sawers, 1994; Kammel et al., 2022]. Hypophosphite enters the cell through FocA and *focA* mutants are insensitive to hypophosphite [Suppmann and Sawers, 1994]. The anaerobic

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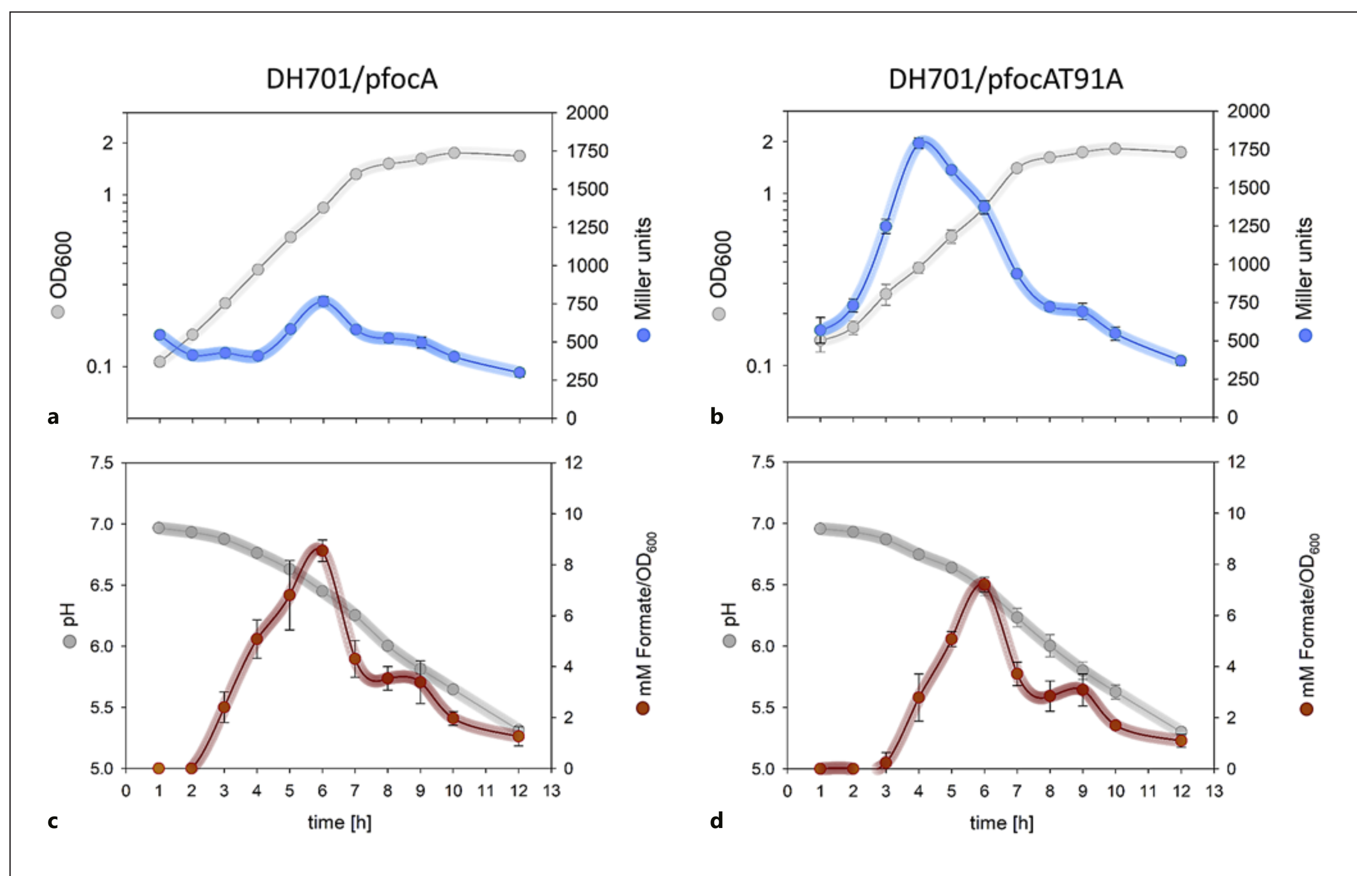

Fig. 3. Strains synthesizing $FocA_{T91A}$ accumulate formate intracellularly. DH701/pfocA, a *focA* mutant synthesizing plasmid-encoded, native FocA (**a**, **c**), and DH701 (*focA*) synthesizing plasmid-encoded $FocA_{T91A}$ (**b**, **d**) were grown anaerobically in glucose-M9 minimal medium. Samples of cells and culture medium were taken every hour to assess changes in *fdhF*$_P$::*lacZ* expression (blue curves), reflecting changes in intracellular formate levels, and the extracellular formate concentration relative to optical density measured at 600 nm (red curves). The following parameters were determined: the optical density (OD_{600}) of the cultures and β-galactosidase enzyme activity (**a**, **b**), and pH and extracellular formate concentration (**c**, **d**). All experiments were performed with three biological replicates.

growth rate of DH701 synthesizing native FocA was reduced by approximately 50% when 0.5-mM hypophosphite was added to the growth medium (Fig. 2c; for percentage reduction of growth rates see online suppl. Table S3). In contrast, the growth rate of the *focA* mutant DH701 lacking any FocA was reduced by only 5% (Fig. 2c). The $FocA_{H209A}$, $FocA_{H209I}$, $FocA_{H209T}$, and $FocA_{H209N}$ variants all showed only a minor reduction in growth rate of approximately 10%, indicating that hypophosphite uptake was severely impaired in DH701 synthesizing these variants (Fig. 2c). The H209F, W, K, and D variants of FocA showed minor reductions in growth rates in the presence of 0.5-mM hypophosphite, which ranged between 4% and 12%. This indicates that no, or only a limited amount of, hypophosphite was taken up by DH701 synthesizing these variants.

T91 Is Essential for Efficient Formate Efflux through FocA

Preliminary evidence has been presented showing that a T91A residue exchange impaired bi-directional formate translocation through FocA, suggesting that T91 has an important function in controlling formate translocation by FocA [Hunger et al., 2014]. To provide more detailed information as to how the T91A exchange affects formate translocation, a comparison of formate-dependent *fdhF*$_p$::*lacZ* expression by DH701 synthesizing native FocA and cells synthesizing $FocA_{T91A}$ throughout anaer-

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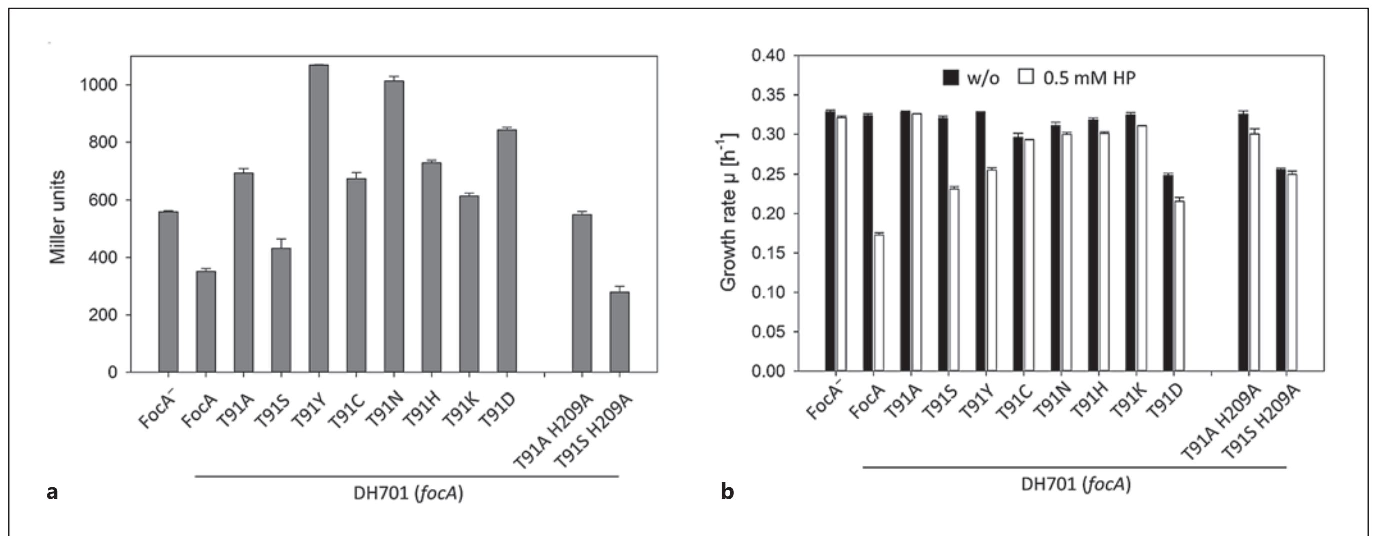

Fig. 4. Threonine or serine at residue position 91 is essential for formate-efflux by FocA. Parameters determined to monitor formate translocation were investigated in the *focA* mutant DH701 (*focA*) and in DH701 transformed with plasmids carrying a *focA* gene encoding the indicated T91 single, and T91-H209 double, amino acid-exchange variants. Strains were grown anaerobically in glucose-M9-minimal medium. **a** Analysis of the formate-induced β-galactosidase enzyme activity after growth of the indicated strains to the late-exponential phase. **b** The sensitivity of the respective strains towards sodium hypophosphite was analysed by determination of anaerobic growth rates in the absence (black histogram) and in the presence of 0.5-mM sodium hypophosphite (white histogram). All experiments were performed with minimally three biological replicates, with each assay performed in duplicate.

obic growth in batch culture was done. It should be noted that the growth rates and final cell densities attained for DH701 synthesizing native FocA or $FocA_{T91A}$, along with their respective pH profiles, glucose consumption, and lactate formation levels, were all comparable (Fig. 3a–d, online suppl. Fig. S2a, b). The data revealed that the formate profiles (reflected as β-galactosidase enzyme activity) for cultures of DH701 synthesizing either native FocA or $FocA_{T91A}$ were significantly different (Fig. 3a, b). High intracellular formate concentration was observed for DH701 synthesizing plasmid-encoded $FocA_{T91A}$ during mid-exponential growth and peaked at a pH of 6.7 (Fig. 3b). In contrast, the peak formate level determined for DH701 synthesizing native FocA was 2.3-fold lower, correlated with a pH of 6.5 in the growth medium, and peaked during the late-exponential phase (Fig. 3a). The formate profile (enzyme activity) of DH701/$FocA_{T91A}$ was more similar to that of the *focA* mutant DH701 [Kammel et al., 2022]. In both strains, the formate level decreased during stationary phase cultivation until only a low level was detectable in late-stationary phase cells (Fig. 3a, b); this was also observed previously for DH701 [Kammel et al., 2022]. The cells clearly take up either undissociated formic acid by passive diffusion across the cytoplasmic membrane, or via another unknown system, and this is ultimately disproportionated to hydrogen and CO_2 by the already induced formate hydrogenlyase complex [Rossmann et al., 1991; Kammel et al., 2022]. These data suggest that cells synthesizing the T91A variant of FocA accumulate formate intracellularly because they are unable to export formate effectively during the exponential phase growth (pH ~7).

After demonstrating that $FocA_{T91A}$ is impaired in formate efflux, next, we determined the impact of introducing different amino acid exchanges at position 91 in FocA on intracellular formate levels. First, the consequences of exchanging T91 for a serine residue were tested (Fig. 4a). After anaerobic growth of DH701 transformed with plasmid pfocAT91S (encoding $FocA_{T91S}$), β-galactosidase enzyme activity was slightly higher than that measured for native FocA but significantly lower than that of DH701 (*focA*). This indicates that serine can replace threonine to allow maintenance of FocA's efflux function in strain DH701, even though it was less effective than native FocA. Exchange of T91 for tyrosine caused strain DH701 to accumulate formate in the cytoplasm, which was reflected by the increased β-galactosidase enzyme activity compared with the *focA* mutant, DH701 (Fig. 4a); the Y91

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variant of FocA thus accumulated more formate intracellularly compared with the *focA* mutant. As FocA variants with a small polar residue and a hydroxyl group at position 91 proved to be functional in formate efflux, we next wanted to test whether the sulfhydryl-group of a cysteine residue was also able to deliver a functional FocA protein. The experiments with DH701 synthesizing $FocA_{T91C}$ indicated formate efflux did not occur (Fig. 4a). Clearly, therefore, the hydroxyl function cannot be replaced by a sulfhydryl group.

A $FocA_{T91N}$ variant was also constructed because a few examples of a T91N exchange are found in the sequence database (Fig. 1b). The $FocA_{T91N}$ variant also proved unable to translocate formate efficiently out of the cytoplasm when synthesized in DH701 (Fig. 4a). Indeed, the formate level (reflected as increased β-galactosidase activity) was almost 2-fold higher compared with DH701 lacking FocA, indicating that formate accumulated intracellularly, as with the T91Y variant. Exchange of T91 for a different H-bonding residue, such as histidine, resulted in synthesis of a FocA variant that forced the strain to accumulate formate intracellularly (Fig. 4a). This FocA variant was apparently also unable to export formate. Finally, the substitution of T91 with charged amino acid residues like lysine and aspartate also resulted in efflux-defective variants of FocA and the $FocA_{T91D}$ variant accumulated formate, albeit less that the level accumulated by the T91Y and N variants (Fig. 4a). In contrast, although the $FocA_{T91K}$ variant was inactive in formate efflux, it did not result in significant intracellular accumulation of formate. These results underscore the importance of a residue with a small hydroxylated side chain at the tip of the Ω-loop in FocA. Note that all of these FocA variants were stably synthesized and inserted into the membrane of DH701, as exemplified for $FocA_{T91A}$ and $FocA_{T91H}$ (online suppl. Fig. S1).

Next, we compared the anaerobic hypophosphite-resistance phenotype of DH701 synthesizing these different T91-variants of FocA (Fig. 4b), which acts as a proxy for estimating effects on formate uptake [Kammel et al., 2022]. As described above, an approximate 50% reduction in anaerobic growth rate was noted when DH701 synthesized native FocA, while when the strain synthesized $FocA_{T91S}$, the growth rate was reduced by 28% in the presence of 0.5-mM hypophosphite (Fig. 4b). This result confirmed that although serine could replace threonine in effecting hypophosphite uptake, it was less efficient compared to threonine. Surprisingly, DH701/$FocA_{T91Y}$ also exhibited partial sensitivity to hypophosphite, whereby a 22% reduction in growth rate due to the addition of 0.5-mM hypophosphite was measured. All of the other amino acid residue exchanges of T91 resulted in a growth-rate reduction in the presence of hypophosphite that was less than 14% (Fig. 4b; online suppl. Table S3). This indicates that hypophosphite uptake was severely impaired when the respective residue replaced T91. Together, these results strongly suggest that a hydroxyl group on the side chain of the residue located at the tip of the Ω-loop is required for FocA to function effectively in formate (and by inference hypophosphite) translocation; however, although the bulkier side chain of tyrosine was non-functional in the formate efflux direction, it was capable of at least partial hypophosphite uptake.

Hydrogen-Bond Formation between Histidine and Threonine Is Important for Formate Translocation

Examination of the meanwhile several hundreds of FNT amino acid sequences in the databases has failed to identify a variant with a serine residue replacing threonine at position 91 [Mukherjee et al., 2017]. This suggests a selective preference for threonine over serine at this position. To test the hypothesis that T91 has a crucial function in formate translocation by FocA, we constructed two variants of FocA, each with two residues exchanged. These variants included $FocA_{T91A/H209A}$ and $FocA_{T91S/H209A}$. The cognate plasmids (see online suppl. Table S1) were freshly transformed into the *focA* mutant DH701 followed by anaerobic cultivation in M9-glucose minimal medium to mid-exponential growth phase. First of all, Western blot analysis was performed to determine whether the FocA derivatives were stably synthesized and inserted in the membrane of DH701 (online suppl. Fig. S1). Although synthesized in lower amounts compared to the synthesis of native FocA from pfocA, $FocA_{T91S/H209A}$ was readily detectable in the membrane fraction of the strain. In contrast, $FocA_{T91A/H209A}$ could only be detected after induction of expression of the cloned *focA* gene by the addition of anhydrotetracycline to the growth medium. This suggested that this FocA variant was made at a very low level in DH701.

Measurement of β-galactosidase enzyme activity for strains expressing the double mutants was then undertaken (Fig. 4a). The results revealed that enzyme activity for $FocA_{T91S/H209A}$ was actually lower than for the *focA* mutant DH701 synthesizing native FocA. $FocA_{T91A/H209A}$, on the other hand, apparently did not excrete formate and displayed comparable β-galactosidase enzyme activity to DH701 (*focA*). The result indicates that when serine replaced T91, and when this was combined with an alanine replacement of H209, then the variant no longer

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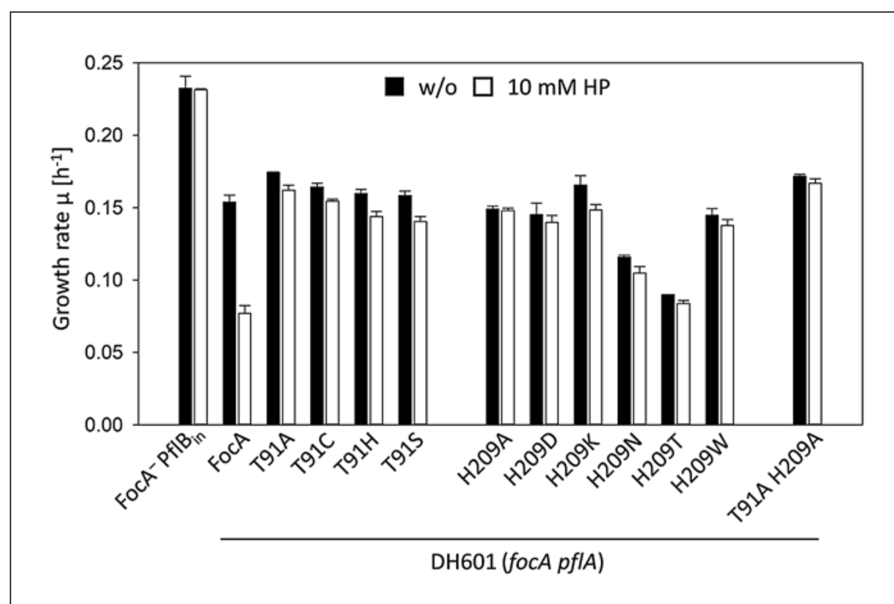

Fig. 5. Both threonine-91 and histidine-209 are essential for efficient translocation of hypophosphite by FocA. Sensitivity towards hypophosphite of strain DH601 (Δ*pflA, focA*) synthesizing the indicated FocA variants was assessed by determining anaerobic growth rates in M9-glucose minimal medium in the absence (black histograms) or presence (white histograms) of 10-mM sodium hypophosphite. The analysis was performed with three biological replicates and repeated twice.

functioned as an exclusive formate efflux protein (compare results with $FocA_{H209A}$ in Fig. 2a). Strain DH701 synthesizing $FocA_{T91A/H209A}$ also failed to efflux formate effectively (Fig. 4a); however, we cannot exclude that this was because of restricted synthesis or stability of this FocA variant. Nevertheless, the result demonstrates that the T91S/A exchanges are dominant over H209A with respect to causing an efflux-negative phenotype and the data suggest that hydrogen-bond formation between histidine and threonine (or serine) is important for controlled formate translocation in the efflux direction.

Both of the FocA double-exchange variants, when synthesized in DH701, failed to show a hypophosphite-sensitive growth phenotype compared to native FocA (Fig. 4b). This indicates that these double-exchange variants were impaired in hypophosphite and, most likely, formate uptake.

T91 and H209 Are Essential for Efficient Hypophosphite Uptake by FocA

Introduction of a mutation in the *pflA* gene, encoding PflB activase, prevents activation of PflB and consequently results in no intracellular production of formate from this, or any other, formate-lyase reaction [Knappe and Sawers, 1990; Heßlinger et al., 1998]. Although active PflB is the principal target of the formate analogue hypophosphite [Suppmann and Sawers, 1994], even when PflB is inactive significantly increasing the concentration of exogenously added hypophosphite nevertheless impairs growth further [Suppmann and Sawers, 1994]. This is possibly a consequence of hypophosphite inhibiting other glycyl-radical enzymes, e.g., anaerobic ribonucleotide reductase. When strain DH601 (*focA,* Δ*pflA*) was grown anaerobically in glucose-M9 medium supplemented with 10-mM sodium hypophosphite and compared to no hypophosphite addition, no difference in the growth rate was observed (Fig. 5; online suppl. Table S3) [Kammel et al., 2021]. In contrast, the same strain transformed with plasmid pfocA (encoding native FocA) showed an approximate 50% reduction in the anaerobic growth rate in the presence of 10-mM hypophosphite compared with no addition; note that even in the absence of added hypophosphite, overexpression of *focA* appears to reduce the anaerobic growth rate by approximately 30% (Fig. 5). Hypophosphite at 10-mM concentration thus severely reduces anaerobic growth, even when PflB is inactive, and hypophosphite causes this effect by entering the cell through FocA. As a consequence, this validates use of this assay system to analyse the effects of amino acid exchanges in the pore of FocA on the protein's ability to translocate hypophosphite in the complete absence of potentially competing intracellular formate.

Determination of the difference in growth rates in the presence versus absence of hypophosphite for DH601 synthesizing the different FocA variants revealed, firstly, that no variant functioned as effectively as native FocA (Fig. 5; online suppl. Table S3). The $FocA_{H209A}$, $FocA_{H209D}$, $FocA_{H209W}$, as well as the double exchange vari-

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ant $FocA_{T91A/H209A}$, all caused a less than 5% reduction in growth rate (Fig. 5; online suppl. Table S3). The other variants, $FocA_{T91A/C/H/S}$, $FocA_{H209K/N/T}$, and $FocA_{T91A\ H209A}$ all caused between a 5% and 10% impairment in the growth of strain DH601. Together, these results indicate that hypophosphite (and presumably by extension also formate) uptake is significantly impaired by any amino acid residue exchange of H209 or T91. Even the conservative exchange of T91 for a serine residue did not allow a significant retention of hypophosphite uptake and underscored the observation made using strain DH701 (*focA*) that, despite the hydroxyl group, the serine residue is less effective than a threonine residue at this position.

Discussion

The results presented in the current study underscore the importance not only of H209, but also of T91 for controlled permeation of formate in vivo through the FocA channel. Moreover, the importance of the hydroxyl group on the side chain of threonine at position 91 for channel function strongly suggests that the interaction between the threonine and histidine residues facilitates bi-directional anion passage through the pore. While it is possible to exchange H209 for a few select amino acid residues that still allow it to function, albeit only in the efflux direction, exchange of T91 for any other residue, except serine, impaired efflux of formate. Indeed, many exchanges caused intracellular accumulation of formate, as well as impaired uptake of both formate and its analogue hypophosphite. While tyrosine with its phenolic hydroxyl group functioned only poorly in the uptake direction, it was completely ineffective in the efflux direction. Thus, T91 is unequivocally important for efficient formate permeation in both directions.

Our recent results in which the histidine residue was exchanged for the non-protonatable residue asparagine resulted in exclusive formate efflux [Kammel et al., 2022]. While a few examples of FNT channels with an asparagine replacing histidine occur naturally, here, we exchanged H209 for several amino acid residues that do not occur naturally. Those with compact polar or non-polar side chains (alanine, isoleucine, and threonine) still permitted efficient formate efflux; however, like $FocA_{H209N}$, these variants were impaired in formate or hypophosphite uptake into cells. These findings further substantiate the recent proposal [Kammel et al., 2022] that a protonatable histidine within the pore is essential for formate permeation in the uptake direction. Of these three residues, only threonine can be an H-bond acceptor or donor, which indicates that anion efflux can be achieved in variants without invoking H-bond formation, although a compact side chain structure is crucial.

When amino acid residues with sterically bulkier side chains, or residues with charged side chains, replaced histidine, then no, or severely impaired permeation of formate (including hypophosphite uptake) in either direction was observed. Together, these results suggest that bulky or charged residues impede the movement of the anion through the narrow constriction sites and hydrophobic core of the pores in the channel, and at the same time delimit the residues that still permit formate efflux [Kammel et al., 2022]. These effects are presumably due to electrostatic repulsion (e.g., aspartate), excessively strong electrostatic anion attraction (e.g., lysine), or steric blockage of the pore (e.g., with phenylalanine or tryptophan). Notably, because only N209 and Q209 variants of FNTs occur naturally, this suggests that the ability of the residue at position 209 on the S-loop to form a hydrogen bond with T91 is important for the correct functionality of FocA, as has been suggested on the basis of structural analyses [Lü et al., 2013; Waight et al., 2013; Hunger et al., 2014].

The threonine residue located at the tip of the Ω-loop in FocA (Fig. 1a) is conserved in all FNT proteins, except in those found in certain species of ascomycete fungi [Mukherjee et al., 2017; Kammel and Sawers, data not shown]. In those few cases, threonine is replaced by an asparagine. The $FocA_{T91N}$ variant constructed and analysed in the current study was inactive in formate permeation and caused intracellular accumulation of the anion. This suggests that in these fungi other amino acid exchanges in the FNT might be necessary to allow anion permeation in the fungal system to occur.

No other amino acid residue, with the exception of serine, could replace threonine effectively at the tip of the Ω-loop in FocA. Despite the serine-exchange variant retaining at least a certain level of native formate efflux and uptake in strain DH701, this was not the case in strain DH601, which only has an inactive PflB and thus makes no intracellular formate. Moreover, when the S91 exchange was combined with an A209 exchange no hypophosphite uptake in strain DH701 (*focA*) was observed. This suggests that formate permeation through this variant is likely also impaired. An appropriately positioned hydroxyl group, aided by the methyl group on the side chain of threonine, at the tip of the Ω-loop that interacts with H209 in FocA is thus important for optimal translocation function. This is supported by the fact that the

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structurally larger tyrosine could only replace threonine at this position poorly.

A detailed analysis of intracellular and extracellular formate profiles throughout the growth phase carried out with a $FocA_{T91A}$ variant showed that cells synthesizing this protein accumulated formate intracellularly, especially in the early phases of growth (see Fig. 3b), indicating impaired efflux of formate. Nevertheless, formate was detected in the growth medium, which suggests that either a further system exits to export the anion or the high permeation coefficient of the undissociated formic acid (pK_a = 3.75) might allow some slow passive diffusion of the acid across the membrane when the internal concentration becomes excessively high. Furthermore, the finding that the $FocA_{T91A}$ variant resulted in formate accumulation intracellularly suggests that native FocA has an important role in equilibrating formate across the membrane during the early stages of anaerobic growth. Replacement of the hydroxyl group of threonine with a thiol (cysteine) proved ineffective and the resulting $FocA_{T91C}$ variant was severely impaired in bi-directional formate permeation. This implies that the ability of the hydroxyl group of threonine to form an H-bond with H209, and to coordinate with water [Lü et al., 2012], is important for formate permeation; the larger sulphur atom is only very weakly electronegative (EN = 2.58) compared with oxygen (EN = 3.44), and so coordinates water poorly.

The water molecule coordinated to T91 has been proposed to recover the proton from formic acid or nitrous acid during uptake of nitrite by NirC [Lü et al., 2012] because results of molecular dynamic studies indicate that a charged molecule cannot pass through the high energetic barrier imposed by the hydrophobicity of the pore [Lv et al., 2013; Atkovska and Hub, 2017]. However, the results presented here indicate that the T91-H_2O interaction is also likely to be important for formate translocation from the cytoplasm into the periplasm, possibly through helping polarize the hydroxyl group and thus aiding H-bond formation with the histidine's imidazole side chain. This would, in turn, imply that in native FocA, the H_2O-T91-H209 H-bond network has a key role in controlling bi-directional anion permeation. Although we cannot as yet propose a mechanism for this permeation, these data suggest that the previously proposed "closed", i.e., the H-bonded conformation, identified in structural analyses might be the important conformation to allow controlled outward or inward anion permeation in vivo. Of course, the caveat to this proposal is that A209 and I209 variants of FocA, which cannot form an H-bond with T91, still allow formate permeation; however, only in the efflux direction. It is conceivable, therefore, that the side chains of alanine and isoleucine are nevertheless sufficiently compact to allow anion passage across the cytoplasmic constriction site (see Fig. 1a). We also recently demonstrated that the soluble N-terminal domain of FocA is essential both for bi-directional formate permeation [Kammel et al., 2021] and for formate efflux by the $FocA_{H209N}$ variant [Kammel et al., 2022]. Because PflB interacts with this domain [Doberenz et al., 2014], we propose that this interaction is required to ensure that the Ω-loop is appropriately positioned near H209 to allow regulation of formate efflux, and at low pH, formate uptake when H209 is protonated. Indeed, the structure of *E. coli* FocA was obtained with a protein lacking a complete N-terminal domain [Wang et al., 2009] and the structure revealed that T91 and H209 were separated by 5.7 Å and consequently not H-bonded (see Fig. 1a); nor was a coordinated water molecule resolved in the structure. Because removal of the N-terminal domain results in an inactive FocA channel [Kammel et al., 2021], this is in accord with our proposal that the interaction network is important for anion permeation. Future studies must now focus on the mechanism of formate permeation and whether, as has been proposed [Lü et al., 2012], a proton-relay within the H-bonding network recaptures the H^+ suggested to be co-translocated with formate in the uptake of the anion, or whether, as has also been alternatively proposed [Helmstetter et al., 2019], that formate: H^+ symport drives anion uptake, without recapture of the H^+.

Materials and Methods

Bacterial Strains, Plasmids, and General Cultivation Conditions

The strains, phage, and plasmids used in this study are listed in online supplementary Table S1. Growth of strains was done anaerobically in an M9 minimal medium [Sambrook et al., 1989], at 37°C, supplemented with 0.8% (wt/vol) glucose as a carbon source [Kammel et al., 2021]. For the investigation of extra- and intracellular formate in the exponential growth phase of DH601/DH701 strains transformed with plasmids carrying genes encoding different FocA variants, growth was done anaerobically in 15-mL Hungate tubes. When metabolic parameters were determined throughout the growth curve (sampled every hour) and the synthesis of various FocA variants was verified by Western blot analysis, cells were cultivated anaerobically in 250 or 500-mL serum bottles.

Where indicated, sodium formate was supplemented to a final concentration of 20 mM and sodium hypophosphite to a final concentration of 0.5 mM (for strain DH701), or 10 mM (for strain DH601) [Kammel et al., 2021, 2022]. When required, antibiotics were added to a final concentration of 50 μg/mL for kanamycin and 100 μg/mL for ampicillin.

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Construction of Plasmids

Plasmid pfocA carrying the native *focA* gene [Kammel et al., 2022] was used as the DNA template for the introduction of mutations in codons 91 and/or 209 of the gene. The oligonucleotides (IDT BVBA, Interleuvenlaan, Belgium) for site-directed mutagenesis are listed in online supplementary Table S2 (for methods, see [Kammel et al., 2022]). All introduced site-specific mutations were verified by DNA sequence analysis of the complete *focA* gene.

Hypophosphite-Sensitivity Test and β-Galactosidase Enzyme Activity Assay

The sensitivity of strains toward the formate analogue hypophosphite was tested by analysis of the growth rates in the exponential growth phase exactly as described [Kammel et al., 2021]. The growth analyses were performed in duplicate with biological triplicates and growth rates are presented with a standard deviation of the mean.

β-Galactosidase enzyme activity was measured and calculated as described [Miller, 1972] but performed with the modifications described [Kammel et al., 2021]. Unless otherwise indicated, all experiments were performed with at least biological triplicates when samples were taken throughout the growth curve. When experiments in which cultures for enzyme activity analysis were harvested in the mid-to-late-exponential phase of growth (OD ~ 0.7–0.9), the analysis was performed in duplicate. The β-galactosidase enzyme activities, presented in Miller units, are given with the standard deviation of the mean.

Analysis of Formate, Lactate, and Glucose Concentrations in the Culture Medium

The analysis of extracellular formic acid, lactic acid, and glucose levels in the culture medium was performed using HPLC exactly as described [Kammel et al., 2022]. The experiments were performed with minimally three biological replicates and data are presented with a standard deviation of the mean.

Verification of Synthesis and Membrane Integrity of FocA Variants

Preparation of membrane fractions for the identification of FocA variants was performed as described [Kammel et al., 2021, 2022]. Solubilized membrane fractions (50-µg protein) were separated by gel electrophoresis using denaturing 12.5% (wt/vol) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate [Laemmli, 1970]. Subsequently, Western blot analysis was carried out using anti-FocA antiserum (diluted 1:1,000) as described [Kammel et al., 2021, 2022].

Computational Tools

To illustrate the locations of T91 and H209 within the FocA pore (PDB structure 3KCU [Wang et al., 2009]) a single protomer was visualized. The distance between the *pros*-N hydrogen on the imidazolium of histidine and the hydroxyl-group of the threonyl residue was determined using PyMOL (The PyMOL Molecular Graphics System, version 2.5, Schrödinger, LLC). The degree of conservation of the amino acids from position 85 to 95 (*E. coli* FocA numbering) in the sequence alignment of 258 annotated FNTs was assessed and displayed using the WebLogo tool (online version 2.8.2) [Crooks et al., 2004].

Statement of Ethics

An ethics statement was not required for this study type, as no human or animal subjects or materials were used.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Michelle Kammel and Oliver Trebbin carried out the experiments. Michelle Kammel and R. Gary Sawers designed the experiments and Michelle Kammel, Oliver Trebbin, and R. Gary Sawers analysed the data. Michelle Kammel and R. Gary Sawers drafted the manuscript and conceived the study. All authors read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Atkovska K, Hub JS. Energetics and mechanism of anion permeation across formate-nitrite transporters. Sci Rep. 2017 Sep;7:12027.

Beyer L, Doberenz C, Falke D, Hunger D, Suppmann B, Sawers RG. Coordinating FocA and pyruvate formate-lyase synthesis in *Escherichia coli*: preferential translocation of formate over other mixed-acid fermentation products. J Bacteriol. 2013 Apr;195:1428–35.

Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. Genome Res. 2004 Jun;14:1188–90.

Doberenz C, Zorn M, Falke D, Nannemann D, Hunger D, Beyer L, et al. Pyruvate formate-lyase interacts directly with the formate channel FocA to regulate formate translocation. J Mol Biol. 2014 Jul;426:2827–39.

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Helmstetter F, Arnold P, Höger B, Petersen LM, Beitz E. Formate-nitrite transporters carrying nonprotonatable amide amino acids instead of a central histidine maintain pH-dependent transport. J Biol Chem. 2019 Jan;294:623–31.

Heßlinger C, Fairhurst SA, Sawers G. Novel keto acid formate-lyase and propionate kinase activities are components of an anaerobic pathway in *Escherichia coli* that degrades L-threonine to propionate. Mol Microbiol. 1998 Jan;27:477–92.

Hopper S, Böck A. Effector-mediated stimulation of ATPase activity by the sigma 54-dependent transcriptional activator FHLA from *Escherichia coli*. J Bacteriol. 1995 May;177:2798–803.

Hunger D, Doberenz C, Sawers RG. Identification of key residues in the formate channel FocA that control import and export of formate. Biol Chem. 2014 Jul;395:813–25.

Kammel M, Hunger D, Sawers RG. The soluble cytoplasmic N-terminal domain of the FocA channel gates bidirectional formate translocation. Mol Microbiol. 2021 Apr;115(4):758–73.

Kammel M, Trebbin O, Pinske C, Sawers RG. A single amino acid exchange converts FocA into a unidirectional efflux channel for formate. Microbiology. 2022 Jan;168(1):001132.

Knappe J, Sawers G. A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. FEMS Microbiol Rev. 1990 Aug;6:383–98.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug;227:680–5.

Lü W, Du J, Schwarzer NJ, Wacker T, Andrade SLA, Einsle O, et al. The formate/nitrite transporter family of anion channels. Biol Chem. 2013 Jun;394:715–27.

Lü W, Du J, Wacker T, Gerbig-Smentek E, Andrade SLA, Einsle O, et al. pH-dependent gating in a FocA formate channel. Science. 2011 Apr;332:352–4.

Lü W, Schwarzer NJ, Du J, Gerbig-Smentek E, Andrade SLA, Einsle O, et al. Structural and functional characterization of the nitrite channel NirC from *Salmonella* Typhimurium. Proc Natl Acad Sci U S A. 2012 Nov;109:18395–400.

Lv X, Liu H, Ke M, Gong H. Exploring the pH-dependent substrate transport mechanism of FocA using molecular dynamics simulation. Biophys J. 2013 Dec;105:2714–23.

Miller JH. Experiments in molecular genetics. NY: Cold Spring Harbor; 1972.

Mukherjee M, Vajpai M, Sankararamakrishnan R. Anion-selective formate/nitrite transporters: taxonomic distribution, phylogenetic analysis and subfamily-specific conservation pattern in prokaryotes. BMC Genomics. 2017 Jul;18:560.

Plaga W, Frank R, Knappe J. Catalytic-site mapping of pyruvate formate lyase: hypophosphite reaction on the acetyl-enzyme intermediate affords carbon-phosphorus bond synthesis (1-hydroxyethylphosphonate). Eur J Biochem. 1988 Dec;178:445–50.

Rossmann R, Sawers G, Böck A. Mechanism of regulation of the formate-hydrogenlyase pathway by oxygen, nitrate and pH: definition of the formate regulon. Mol Microbiol. 1991 Nov;5:2807–14.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. NY: Cold Spring Harbor; 1989.

Sargent F. The model [NiFe]-hydrogenases of *Escherichia coli*. Adv Microb Physiol. 2016 Mar;68:433–507.

Sawers RG. Formate and its role in hydrogen production in *Escherichia coli*. Biochem Soc Trans. 2005 Feb;33:42–6.

Sui H, Han BG, Lee JK, Walian P, Jap BK. Structural basis of water-specific transport through the AQP1 water channel. Nature. 2001 Dec;414:872–8.

Suppmann B, Sawers G. Isolation and characterisation of hypophosphite-resistant mutants of *Escherichia coli*: identification of the FocA protein, encoded by the *pfl* operon, as a putative formate transporter. Mol Microbiol. 1994 Mar;11:965–82.

Waight AB, Czyzewski BK, Wang DN. Ion selectivity and gating mechanisms of FNT channels. Curr Opin Struct Biol. 2013 Aug;23:499–506.

Waight AB, Love J, Wang DN. Structure and mechanism of a pentameric formate channel. Nat Struct Mol Biol. 2010 Jan;17:31–7.

Wang Y, Huang Y, Wang J, Cheng C, Huang W, Lu P, et al. Structure of the formate transporter FocA reveals a pentameric aquaporin-like channel. Nature. 2009 Nov;462:467–72.

Wiechert M, Beitz E. Mechanism of formate-nitrite transporters by dielectric shift of substrate acidity. EMBO J. 2017 Apr;36:949–58.

Wu B, Rambow J, Bock S, Holm-Bertelsen J, Wiechert M, Soares AB, et al. Identity of a *Plasmodium* lactate/H+ symporter structurally unrelated to human transporters. Nat Commun. 2015 Feb;6:6284.

Zeng JM, Hapuarachchi SV, Shafik SH, Martin RE, Kirk K, van Dooren GG, et al. Identifying the major lactate transporter of *Toxoplasma gondii* tachyzoites. Sci Rep. 2021 Mar;11(1):6787.

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