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Amine Ali Chaouche, Laetitia Houot, Denis Duché, Chantal Iobbi-Nivol, Marie-Thérèse Giudici-Ortoni, et al.. The Tol-Pal system of *Escherichia coli* plays an unexpected role in the import of the oxyanions chromate and phosphate. *Research in Microbiology*, 2022, 173 (8), pp.103967. 10.1016/j.resmic.2022.103967 . hal-03783277

HAL Id: hal-03783277

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Original Article

The Tol–Pal system of *Escherichia coli* plays an unexpected role in the import of the oxyanions chromate and phosphate

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ARTICLE INFO

Article history:

Received 25 February 2022

Accepted 24 May 2022

Available online 1 June 2022

Keywords:

Tol–Pal system

Phosphate import

Oxyanions uptake

Chromate resistance

ABSTRACT

Chromate is a toxic metal that enters bacteria by using oxyanion importers. Here, we show that each mutant of the Tol–Pal system of *Escherichia coli* exhibited increased chromate resistance. This system, which spans the cell envelope, plays a major role in envelope integrity and septation. The $\Delta tolQR$ mutant accumulated three-fold less chromate than the wild-type. Addition of phosphate but not sulfate to rich medium drastically reduced chromate toxicity and import in the wild-type strain. Furthermore, the intracellular concentration of free inorganic phosphate was significantly reduced for the $\Delta tolR$ mutant in comparison to the wild-type strain. Moreover, extracellular labeled phosphate was significantly less incorporated into the $\Delta tolR$ mutant. Finally, two distinct TolQR mutant complexes, specifically affected in Tol–Pal energization without affecting the TolQRA complex structure, did not complement the $\Delta tolQR$ mutant for inorganic phosphate accumulation. We thus propose that, while the Pst system is well known to import inorganic phosphate, the Tol–Pal system participates to phosphate uptake in particular at medium to high extracellular phosphate concentrations. Since mutations disabling the Tol–Pal system lead to pleiotropic effects, chromate resistance and reduced inorganic phosphate import could occur from an indirect effect of mutations in components of the Tol–Pal system.

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1. Introduction

Chromium is a non-essential but toxic metal for cells [1]. The most toxic form is the soluble hexavalent form, chromate (Cr(VI)). Bacteria cope with chromate mainly by using efflux pumps to extrude it and by extracellularly reducing Cr(VI) into Cr(III) [2–5]. As Cr(III) is less soluble than Cr(VI), it cannot enter the cells. Because Cr(VI) induces reactive oxygen species (ROS) and DNA lesions, bacteria facing Cr(VI) trigger repair mechanisms, including the oxidative stress response and the SOS system [2,6]. Certain bacteria can resist high Cr(VI) concentrations, whereas others, including *Escherichia coli*, are less resistant [4,6], most likely

because bacteria can or cannot encounter Cr(VI) in their natural environment. It is still largely unclear how Cr(VI) enters the bacteria. To date, it has been proposed that Cr(VI) uses sulfate importers to penetrate into the cytoplasm [1,7]. Indeed, chromate and sulfate are homologous oxyanions. In the present study, we propose that chromate is mainly imported into *E. coli* by an unsuspected pump probably involving the Tol–Pal system. This system also appears to play a major role in the import of the inorganic phosphate oxyanion (Pi) but not in that of sulfate oxyanion. Phosphate, as sulfate, are essential elements for life, and three transporters have been proposed for phosphate uptake: the well-known Pst system as well as the PitA and the PitB transporters [8,9]. The PstSCAB is a high-affinity ABC transporter involved specifically in Pi uptake. This system is regulated by the PhoR/PhoB two-component system. Both PitA and PitB are low-affinity Pi transporters but PitA is positively regulated by Zn(II) and, surprisingly, by low Pi concentrations, whereas *pitB* gene expression is repressed under limiting Pi conditions [9]. More recently, it has been proposed that the main role of PitA and PitB is not Pi transport but rather that of

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metal ion import and export [10]. Therefore, a low-affinity transporter of Pi, able to function under medium to high concentrations of Pi, remains to be discovered. Intracellular Pi concentration is high in *E. coli* (up to 10 mM or even more); in the lumen of the intestine, the usual *E. coli* niche, the phosphate concentration fluctuates from low to high according to the intestinal segment considered and the diet [11,12].

The core components of the Tol–Pal system comprise five proteins. The TolA, TolQ, and TolR are inner membranous proteins, and the TolQR complex forms a proton channel to energize the system [13]. Pal is a lipoprotein anchored to the outer membrane; it can bind both the peptidoglycan (PG) and the periplasmic TolB protein. Both TolA and Pal interact by using the proton-motive force (PMF) mediated by TolQR [14]. The Tol–Pal complex is mandatory to sustain the appropriate structure of the cell envelope [15]. Mutants of the Tol–Pal system are sensitive to detergents, form filaments in low-osmolarity media, and are resistant to group A colicins as well as to filamentous phage uptake [16–18]. Recently, the Tol–Pal system has been shown to accumulate to the septal region during cell division and to orchestrate outer membrane constriction and septal PG synthesis [13,19–21]. Here, we show that, similar to the homologous TonB system which actively transports vital nutrients [22], the Tol–Pal system is most probably involved directly or indirectly in the import of phosphate and, incidentally, toxic chromate.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

Table 1 shows the *E. coli* strains and plasmids. Strains were routinely grown in Lysogeny Broth (LB) medium at 37 °C under shaking (145 rpm), and growth was determined

spectrophotometrically by monitoring changes in the optical density at OD₆₀₀ compared to the same sterile medium. Single-gene knockout MG1655 mutants were obtained by P1 transduction [23], using a phage lysate developed in the corresponding strain from the Keio collection [24]. Transductants were selected on kanamycin-supplemented medium, and the kanamycin cassette (*kan*) was removed using the FLP expression plasmid pCP20 as previously described [25]. When necessary, Kan^S deletion mutants were then subjected to a new round of transduction to introduce a new deletion. Deletions were confirmed by PCR experiments according to the recommended strategy [25].

Plasmids *ptolQR*, *ptolQT145AR* and *ptolQRD23C* [18] were used for complementation experiments.

2.2. Chromate resistance assays

The *E. coli* pre-cultures grown overnight on LB plates were suspended in LB medium and used to inoculate fresh LB medium to an initial OD₆₀₀ = 0.1; cells were grown until an OD₆₀₀ = 1. Subsequently, 10 µL of 10-fold serial dilutions were spotted on LB agar (17 g L⁻¹) plates with or without potassium chromate (K₂CrO₄), L-arabinose (0.02%), magnesium sulfate (MgSO₄), phosphate buffer (Na₂HPO₄/NaH₂PO₄ or K₂HPO₄/KH₂PO₄, pH 7.2), sodium molybdate (Na₂MoO₄), or sodium tungstate (Na₂WO₄), and the plates were incubated at 37 °C for 24 h.

2.3. Preparation of soluble extracts

The wild-type and mutant *E. coli* pre-cultures grown overnight on LB plates were resuspended in LB medium and used to inoculate fresh LB medium to an initial OD₆₀₀ = 0.1; cells were grown on LB medium until reaching an OD₆₀₀ = 0.5. For chromate-phosphate competition experiments, the LB medium was supplemented

Table 1
Strains and plasmids used in this study.

<i>E. coli</i> strains or plasmids	Genotype or description	Source/Reference
W3110 WT	F ^λ IN(<i>rrmD-rrnE</i>)1 <i>rph-1</i>	[26]
W3110 Δ <i>tolQ</i>	W3110 strain deleted of <i>tolQ</i> gene	[19]
W3110 Δ <i>tolR</i>	W3110 strain deleted of <i>tolR</i> gene	[19]
W3110 Δ <i>tolQR</i>	W3110 strain deleted of <i>tolQ</i> and <i>tolR</i> genes	[19]
W3110 Δ <i>tolA</i>	W3110 strain deleted of <i>tolA</i> gene	[19]
W3110 Δ <i>tolB</i>	W3110 strain deleted of <i>tolB</i> gene	[19]
W3110 Δ <i>pal</i>	W3110 strain deleted of <i>pal</i> gene	[19]
W3110 Δ <i>exbD</i>	W3110 strain deleted of <i>exbD</i> gene	This study
W3110 Δ <i>tonB</i>	W3110 strain deleted of <i>tonB</i> gene	This study
W3110 Δ <i>tonB</i> Δ <i>exbD</i>	W3110 strain deleted of <i>tonB</i> and <i>exbD</i> genes	This study
MG1655 WT	K-12 F ⁻ λ ⁻ <i>ilvG</i> ⁻ <i>rfb-50</i> <i>rph-1</i>	[27]
MG1655 Δ <i>tolR</i>	MG1655 strain deleted of <i>tolR</i> gene	This study
MG1655 Δ <i>pitA</i> Δ <i>pitB</i>	MG1655 strain deleted of <i>pitA</i> and <i>pitB</i> genes	This study
MG1655 Δ <i>pitA</i> Δ <i>pitB</i> Δ <i>tolR</i>	MG1655 strain deleted of <i>pitA</i> , <i>pitB</i> and <i>tolR</i> genes	This study
MG1655 Δ <i>pitA</i> Δ <i>pitB</i> Δ <i>pstA</i>	MG1655 strain deleted of <i>pitA</i> , <i>pitB</i> and <i>pstA</i> genes	This study
MG1655 Δ <i>pitA</i> Δ <i>pitB</i> Δ <i>pstA</i> Δ <i>tolR</i>	MG1655 strain deleted of <i>pitA</i> , <i>pitB</i> , <i>pstA</i> and <i>tolR</i> genes	This study
MG1655 Δ <i>cysW</i>	MG1655 strain deleted of <i>cysW</i> gene	This study
BW25113 WT	F- LAM- <i>rrnB3</i> DElacZ4787 <i>hsdR514</i> DE(<i>araBAD</i>)567 DE(<i>rhaBAD</i>)568 <i>rph-1</i>	[25]
BW25113 Δ <i>tolQ</i>	BW25113 strain deleted of <i>tolQ</i> gene	[24]
BW25113 Δ <i>ybgF</i>	BW25113 strain deleted of <i>ybgF</i> gene	[24]
BW25113 Δ <i>pitA</i>	BW25113 strain deleted of <i>pitA</i> gene	[24]
BW25113 Δ <i>pitB</i>	BW25113 strain deleted of <i>pitB</i> gene	[24]
BW25113 Δ <i>pstA</i>	BW25113 strain deleted of <i>pstA</i> gene	[24]
pOK12	Vector with the p15A origin of replication, Kan ^R	[28]
pOK12- <i>tolA</i>	<i>tolA</i> sequence cloned into pOK12	[18]
pOK12- <i>pal</i>	<i>pal</i> sequence cloned into pOK12	[18]
pCP20	FLP expression plasmid, Cm ^R , Amp ^R	[25]
pBAD/HisC	pBR322-derived expression vector, L-arabinose inducible, Amp ^R	Invitrogen
<i>ptolQR</i>	pBAD/HisC carrying the <i>E. coli</i> <i>tolQ</i> gene fused to an HA tag and the <i>tolR</i> gene, Amp ^R	[18]
<i>ptolQT145AR</i>	<i>ptolQR</i> bearing a T-to-A substitution at position 145 in TolQ, Amp ^R	[18]
<i>ptolQRD23C</i>	<i>ptolQR</i> bearing a D-to-C substitution at position 23 in TolR, Amp ^R	[19]

with potassium chromate or phosphate buffer at the indicated final concentration. After 2 h of growth at 37 °C under shaking (145 rpm), the cell cultures were recovered by centrifugation. The bacterial pellets were washed 3 times with 20 mM Hepes buffer (pH 7.2) and subsequently resuspended with Hepes buffer and lysed using the French press (once at 700 psi). The lysates were centrifuged for 15 min at 5800 g to remove cell debris and unbroken bacteria, and the supernatants were ultra-centrifuged for 1 h at 118,000 g. The resulting supernatants corresponded to soluble intracellular extracts. The protein concentrations of the soluble extracts were determined by Bradford assays (BIO-RAD).

2.4. Microscopic observation

Before the preparation of soluble cell extracts as described in the previous paragraph, 20 µL of each cell suspension were introduced within the microscope slide bridge. Cells were observed with a 40× phase contrast objective.

2.5. Total chromium quantification by optical emission spectrometry-inductively coupled plasma analysis (ICP-OES)

The chromium content of soluble extracts was determined using an iCAP-6000 ICP-OES analyzer. The amount of Cr atoms for each sample was normalized with the protein concentration, and three biological replicates were analyzed as indicated in the figure. Pure chromate solution (K₂CrO₄) was used for calibration.

2.6. Intracellular Pi quantification

The free Pi present in soluble extracts was quantified using BIOMOL GREEN™ reagent (Enzo Life Science) based on malachite labeling. This method represents a simple and convenient approach for colorimetric phosphate quantitation by reading the absorbance at 620 nm. Briefly, standards were prepared by dilution of a (K₂HPO₄/KH₂PO₄) stock solution to obtain final Pi concentrations of 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, and 40.0 µM. To be within the detectable range of the Pi assay, the soluble extracts were 20-fold diluted before analysis. The Pi assay was performed in 96-wells plates by adding 100 µL of Biomol Green to each well containing 50 µL of standard or diluted soluble fraction. After incubation at room temperature for 25 min, absorbance was measured at 620 nm with the Spark® TECAN equipment. The amount of Pi for each sample was normalized with the protein concentration. Three biological replicates were analyzed, as indicated in the figure.

2.7. Labeled Pi import quantification

Bacteria were grown in triplicate in 2 mL LB broth. At OD₆₀₀ = 0.1, 20 µCi of ³²Pi (PerkinElmer) were added to each sample, and the cultures were incubated at 37 °C for 16 h under agitation. Subsequently, 1.7 mL of each bacterial culture was centrifuged at 6800 g for 5 min at 4 °C. To remove loosely bound ³²Pi, cell pellets were washed three times by resuspension in 1.7 mL of Hepes buffer (Hepes 20 mM, 2 mM MgCl₂), followed by centrifugation at 6800 g for 5 min. Cells were resuspended in 1.7 mL of Hepes buffer, and 100 µL of cell suspensions was used for the determination of the OD₆₀₀. Cells lysis was performed by freezing in liquid nitrogen and thawing for 15 min at 37 °C, followed by incubation for 15 min with 1× CellLytic B reagent supplemented with benzonase and R-lysozyme, as recommended by the manufacturer (Sigma–Aldrich). Unbroken cells were removed by centrifugation at 15,300 g for 10 min, and membranes were pelleted by ultra-centrifugation (125,000 g during 45 min). The supernatants corresponding to the soluble cell fractions were collected, and

radioactive levels were quantified using a liquid scintillation counter (Hidex 300 SL). Nucleic acid concentrations of each sample were determined by measuring the absorbance signal at 260 nm (BioSpec-nano spectrophotometer, Shimadzu) and used to normalize the radioactivity signal.

3. Results

3.1. Disruption of the Tol–Pal system of *E. coli* increases sensitivity to chromate

We previously isolated the *Shewanella algidipiscicola* strain H1 which is able to grow in the presence of high chromate (Cr(VI)) concentrations [26]. In preliminary experiments, we observed that overproduction of the TolQR homolog of the strain H1 in *E. coli* increases sensitivity to chromate. The TolQR complex from strain H1 could interact with the Tol–Pal system of *E. coli* and this effect could derive from an activation either of the Tol–Pal system or, on the opposite, an inactivation of this system. To answer this question, wild-type and the *E. coli* Δ tolQR strains were grown aerobically at 37 °C in rich medium (LB) and 10-fold serial dilutions were then spotted on LB plates with or without 1 mM Cr(VI). LB medium was used to minimize side effects including filamentation and cell division defects [19,21]. Fig. 1A shows that, after 24 h of incubation, the wild-type and Δ tolQR strains grew similarly on LB plates, as expected. In contrast, in the presence of 1 mM Cr(VI), the wild-type strain did not grow, whereas the Δ tolQR strain grew similarly in the presence as well as in the absence of Cr(VI). This result clearly indicates that the Δ tolQR strain is resistant to 1 mM Cr(VI), suggesting that inactivation of the Tol–Pal system allows Cr(VI) resistance. Using optical microscopy, we confirmed that the size and the shape of the wild-type and Δ tolQR cells were similar in the absence of chromate. In the presence of Cr(VI), the Δ tolQR strain behaved as in the absence of Cr(VI) whereas the wild-type bacteria formed filaments showing that the cells are strongly affected by Cr(VI) (Fig. S1A–B), as described by Ackerley et al. in the case of the WT strain [6]. We tested the effect of deletion of each gene of the Tol–Pal system (Fig. 1A). Strikingly, the Δ tolA, Δ tolB, Δ tolQ, Δ tolR, and Δ pal strains grew similarly in the presence or absence of 1 mM Cr(VI), a chromate concentration deleterious for the wild-type strain. These results demonstrated that inactivation of the Tol–Pal system by deleting any *tol-pal* gene strongly increases the Cr(VI) resistance of *E. coli*. Furthermore, production either of the inner membrane TolA protein in the Δ tolA strain or of the outer membrane Pal lipoprotein in the Δ pal mutant decreased chromate resistance, as expected (Fig. 1B). In summary, the loss of function of this system leads to high chromate resistance.

3.2. Import of chromate is decreased in the Δ tolQR mutant

It is well-known that Cr(VI) resistance is due to either chromate export by an efflux pump, by rapid reduction of Cr(VI) into Cr(III), or both [3,4,6]. Obviously, the oxidative stress induced by Cr(VI) takes place mainly after Cr(VI) has entered into the cell [1]. To confirm that the Tol–Pal system is probably involved in the import of Cr(VI) into *E. coli*, the Δ tolQR and the wild-type strains were grown in the presence or absence of 1 mM Cr(VI) for 2 h in rich medium and, after preparation of the soluble cell fractions, the concentration of total chromium was measured by inductively coupled plasma-optical emission spectrometry (ICP-OES). Fig. 2 shows that the Cr concentration, relative to the concentration of soluble proteins, was three-fold lower in the Δ tolQR strain in comparison to the wild-type strain, indicating that the import of Cr(VI) is strongly reduced when the Tol–Pal system is inactivated. TolQR energizes the system and allows TolA–Pal interaction. Interestingly, the

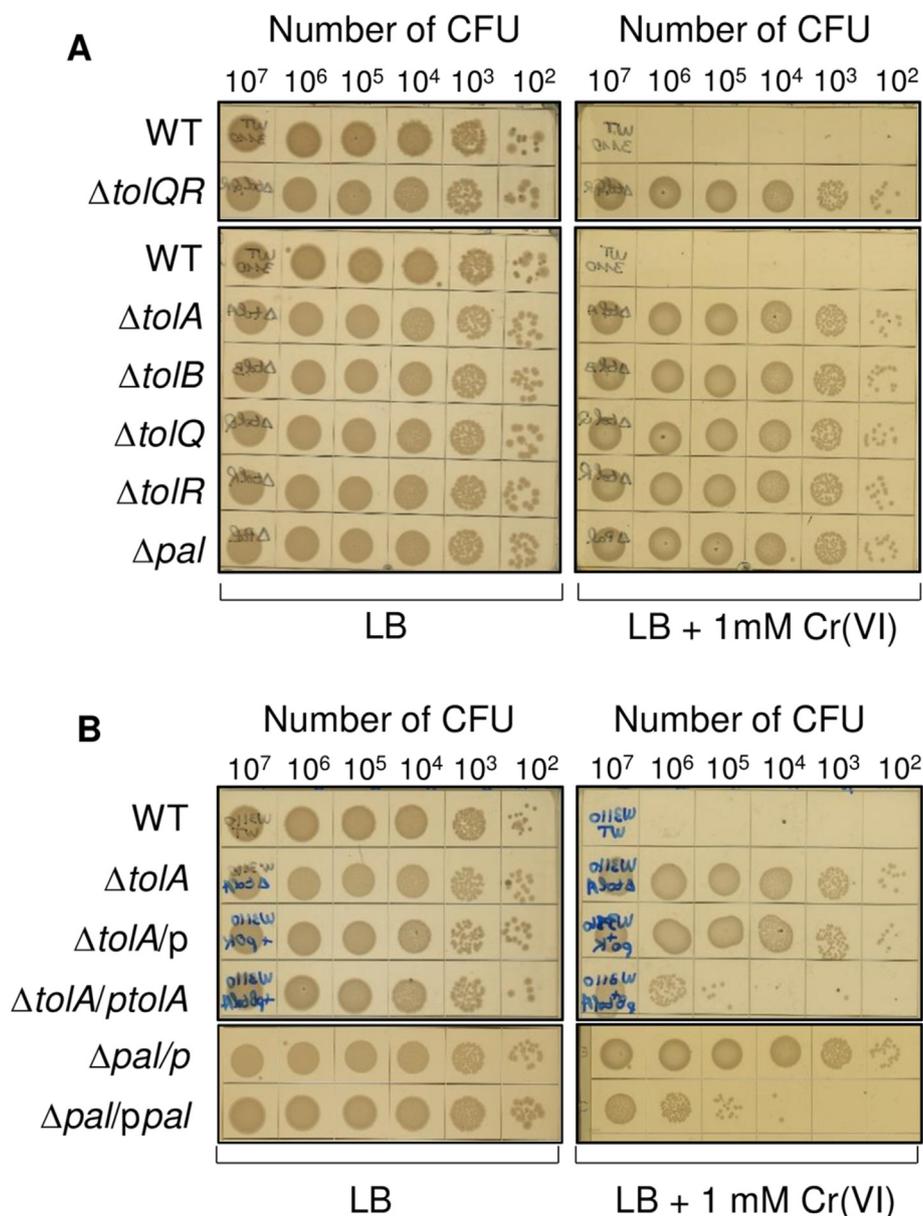


Fig. 1. The *tol pal* genes are associated with sensitivity to chromate. (A) Chromate sensitivity of the W3110 wild-type strain (WT) and Tol–Pal mutants was evaluated. 10-fold serial dilutions of the corresponding cultures were spotted on LB agar with or without 1 mM Cr(VI) before incubation at 37 °C for 24 h. (B) The effect of complementation of the $\Delta tolA$ and Δpal in chromate sensitivity was controlled. 10-fold serial dilutions of the W3110 WT strain, the $\Delta tolA$ mutant, the $\Delta tolA$ strain containing either the pOK12 vector (p) or *ptolA* plasmid, the Δpal strain containing either the pOK12 vector (p) or the *ppal* plasmid were spotted on LB agar with or without 1 mM Cr(VI) before incubation at 37 °C for 24 h. Pictures of the plates in panels A and B are representative of at least three independent experiments.

TolQR_{D23C} variant is stable and forms stable Tol–Pal complexes but the mutant is unable to promote Tol–Pal activation [27–29]. We thus tested whether energization is required for cell Cr(VI) sensitivity. While the $\Delta tolQR$ strain became sensitive to Cr(VI) when the TolQR complex was overproduced from *ptolQR*, the overproduction of TolQR_{D23C} from *ptolQR*_{D23C} did not alter Cr(VI) resistance of the $\Delta tolQR$ strain. This result strongly suggests that energization of the Tol–Pal system is required for Cr(VI) import (Fig. 3).

As the ExbBD and TonB proteins are homologous to TolQR and TolA in *E. coli*, we checked whether deletion of *exbD* or *tonB* conferred chromate resistance. Fig. S2A shows that the deleted strains behaved as the WT strain, indicating that the ExbBD–TonB system is not involved in chromate uptake. Finally, as the sulfate importer has been proposed to allow Cr(VI) entrance in bacteria [1,7], we tested the effect of the deletion of *cysW*, a key component

of the sulfate/thiosulfate CysPTWA ABC-transporter of *E. coli* [30]. The deleted strain was not resistant to Cr(VI) as the wild-type strain, showing that, at least in *E. coli*, CysPTWA is not the main Cr(VI) importer (Fig. S2B).

3.3. Competition between chromate and oxyanions of the same family

The fact that the Tol–Pal system could be involved in the import of chromate is intriguing because chromate is rarely found in the natural surroundings of *E. coli* and is toxic for this strain [6]. We hypothesize that the Tol–Pal system is involved in the import of an essential oxyanion structurally related to chromate, such as sulfate or phosphate. However, importers of sulfate and phosphate are well-known, but transporter redundancy often occurs when the

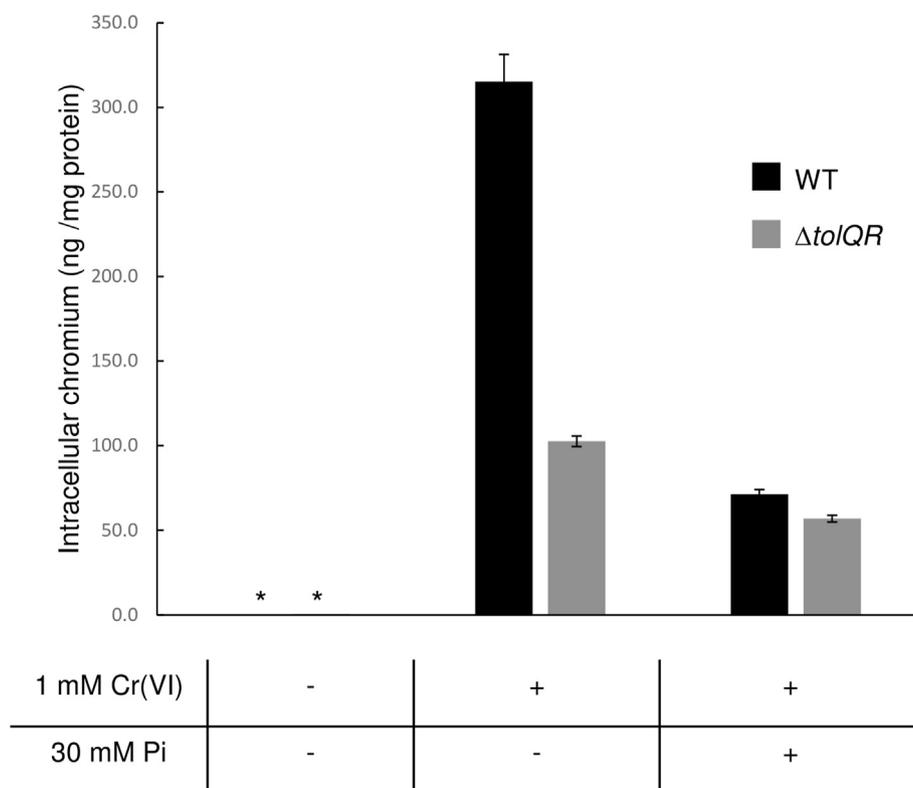


Fig. 2. Intracellular concentration of chromium in the presence or absence of a high concentration of extracellular Pi. Intracellular chromium concentration was evaluated by ICP-OES analysis for the W3110 WT strain and the $\Delta tolQR$ mutant challenged with 1 mM Cr(VI) in the presence or not of 30 mM Pi (K_2HPO_4/KH_2PO_4 , pH 7.2) for 2 h. (*) indicates that values are below the detection level. Error bars represent the standard deviation of the mean from three independent experiments.

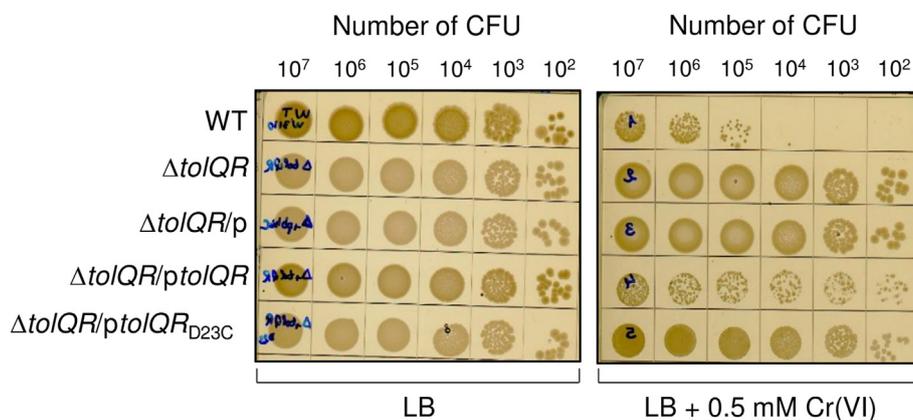


Fig. 3. A functional TolQR complex is required in Cr(VI) sensitivity. Chromate sensitivity of the W3110 WT strain, the $\Delta tolQR$ mutant, the $\Delta tolQR$ containing either pBAD/HisC vector (p), *ptolQR* or *ptolQR_{D23C}* plasmid was evaluated. 10-fold serial dilutions of the corresponding cultures were spotted on LB agar with or without 0.5 mM Cr(VI) and 0.02% L-arabinose before incubation at 37 °C for 24 h. Pictures of the plates are representative of at least three independent experiments.

compound is essential for cell survival or growth. To check this possibility, we carried out competition experiments between chromate and sulfate or phosphate. If competition occurs, the toxicity of chromate must decrease, when concentration of competitor increases. Fig. 4A shows that the addition of 1 mM chromate still killed the wild-type strain in the absence (Fig. 1) or presence of high concentrations of sulfate (Fig. 4A). In contrast, increasing concentrations of phosphate from 5 to 20 mM in the presence of 1 mM chromate restored the growth of the wild-type strain (Fig. 4B). These results were obtained whether the phosphate counterion was Na⁺ (Fig. 4B) or K⁺ (Fig. S3), showing that the counterion plays no role in the process. Moreover, in the presence

of 20 mM phosphate, the growth of the wild-type strain was similar to that of the $\Delta tolQR$ strain in the presence of chromate (Fig. 4B). These results strongly suggest that the Tol–Pal system plays a key role in the import of phosphate but not in that of sulfate.

This result was confirmed by ICP-OES. In the presence of 30 mM phosphate, the intracellular concentration of Cr dropped more than four-fold in the wild-type strain (Fig. 2), and the wild-type cell morphology was restored (Fig. S1C). The addition of 30 mM phosphate decreased less than twice the already low intracellular concentration of Cr in the $\Delta tolQR$ strain. Moreover, the Cr concentrations in the presence of phosphate were close in both the wild-type and $\Delta tolQR$ strains (Fig. 2). These data reinforce the idea

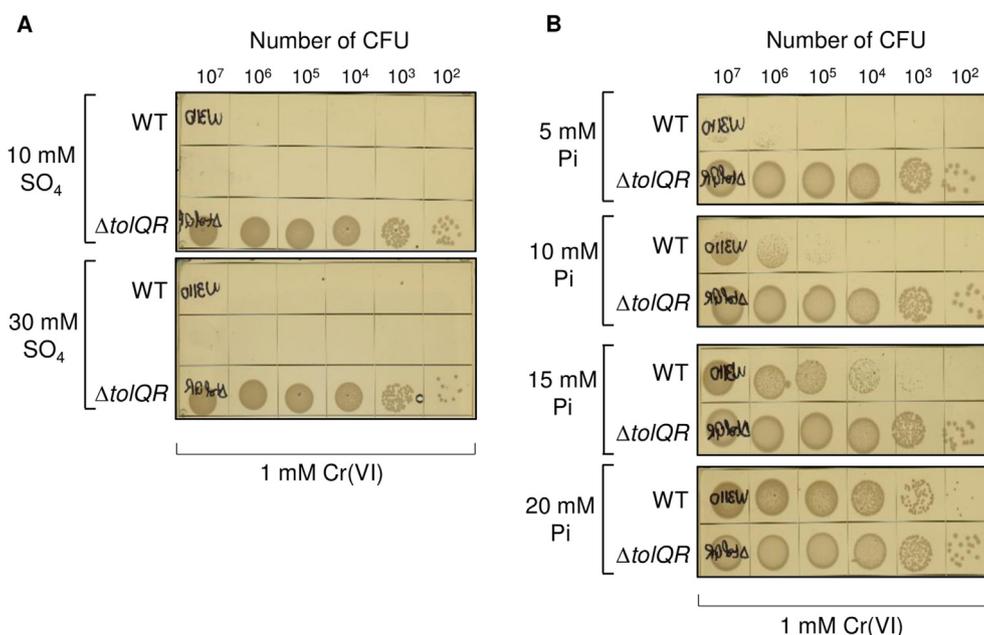


Fig. 4. Effect of addition of sulfate and phosphate on Cr(VI) sensitivity. 10-fold serial dilutions of the W3110 WT strain and the ΔtolQR mutant were spotted on LB agar with 1 mM Cr(VI) and increasing concentrations of (A), sulfate (MgSO_4) or (B), phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2), and incubated at 37°C during 24 h. Pictures of the plates in panels A and B are representative of at least three independent experiments.

that import of chromate and phosphate, at medium to high concentrations, required the presence of the Tol–Pal system. Unfortunately, direct measurement of the intracellular concentration of phosphate is impossible using ICP-OES because several cellular compounds, including ATP, DNA, and RNA, contain phosphorus, and this method detects all phosphorus forms. It is also important to note that all experiments were carried out in LB medium, which contains at least 2 mM of Pi [31].

Molybdate and tungstate also belong to the same family of oxyanions, but they are found as trace elements in natural environments, and a specific high-affinity ABC transporter for molybdate is present in *E. coli* [32,33]. Nevertheless, competition experiments were carried out using a very high and thus non-physiological concentration of molybdate or tungstate (10 mM for each). In the presence of 1 mM chromate, 10 mM of either molybdate or tungstate partially restored the growth of the wild-type strain (Fig. S4). This result shows a competition between chromate and molybdate or tungstate and thus confirms that the Tol–Pal complex is involved in oxyanion import.

3.4. Analysis of the potential role of the known phosphate importers in chromate resistance of *E. coli*

To determine whether the PitAB and Pst systems, known to import phosphate [8,9], might also be chromate importers, we constructed a strain deleted of the *pitA*, *pitB*, and *pstA* genes. As controls, we constructed a ΔtolR strain and the quadruple ΔpitA ΔpitB ΔpstA ΔtolR mutant strain. Fig. 5 shows that, after 24 h of incubation, the wild-type and the mutant strains showed similar growth on LB plates. As expected, in the presence of 1 mM chromate, the wild-type strain was killed, whereas the ΔtolR and the quadruple deletion strains grew as in the absence of chromate. However, for the ΔpitA ΔpitB ΔpstA mutant strain, growth was weak in the presence of chromate, showing that chromate is mainly imported by the Tol–Pal complex under our experimental conditions. In addition, ΔpitA , ΔpitB and ΔpstA simple mutants were tested successfully (Fig. S5). Since *ybgF* gene is part of the *tolB-pal-*

ybgF operon we tested whether deletion of *ybgF* increased cell survival in the presence of Cr(VI) . The results (Fig. S5) indicate that absence of *ybgF* gene did not change the Cr(VI) sensitivity of the strain.

3.5. Import of phosphate is decreased in the ΔtolR strain

If the Tol–Pal system is involved in the import of Pi, inactivation of the system could lead to a lower concentration of free phosphate in the mutant cells in comparison to the wild-type. To measure the intracellular Pi concentrations, we used a robust colorimetric free-phosphate detection assay based on malachite labeling [34]. After overnight growth, the free intracellular Pi concentration of the ΔtolR strain was twice lower than that of the wild-type strain (Fig. 6). This result is in good agreement with the fact that the Tol–Pal system plays a key role in phosphate uptake. Surprisingly, the ΔpitA ΔpitB ΔtolR strain showed only a slight decrease of free Pi concentration than that of the ΔtolR strain, and in the ΔpitA ΔpitB mutant, the level of intracellular Pi was similar to that of the wild-type (Fig. 6). The role in phosphate import of the PitA and PitB systems is thus minor under our experimental conditions in which growth occurred in rich medium.

After entry into the cell, Pi is mainly incorporated into phosphorus-containing molecules such as ATP, DNA, RNA, or phospholipids. Fig. 7 shows that 55% more ^{32}P was measured in the soluble extract of the wild-type strain relatively to that of the ΔtolR strain after overnight growth in the presence of extracellular ^{32}P . This result indicates that the labeled phosphorus is significantly less retrieved in the ΔtolR mutant in comparison to the wild-type strain, thus confirming that the Tol–Pal complex plays a key role in Pi uptake.

3.6. Activation of the Tol–Pal system is required for Pi import

The TolQR complex forms a molecular stator converting the PMF into mechanical movements activating the Tol–Pal system [27,28]. Point mutations in the TolQ subunit ($\text{TolQ}_{\text{T145A}}$) or in the TolR

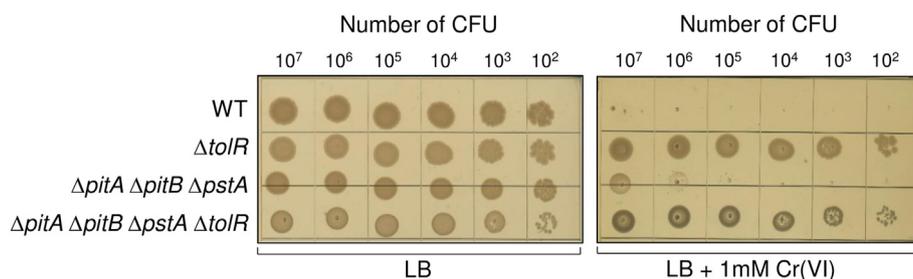


Fig. 5. Effect of deletion of genes involved in Pi uptake on Cr(VI) sensitivity. 10-fold serial dilutions of the MG1655 WT strain, the $\Delta tolR$, the $\Delta pitA \Delta pitB \Delta pstA$, and the $\Delta pitA \Delta pitB \Delta pstA \Delta tolR$ mutant were spotted on LB agar with or without 1 mM Cr(VI) before incubation at 37 °C for 24 h. Pictures of the plates are representative of at least three independent experiments.

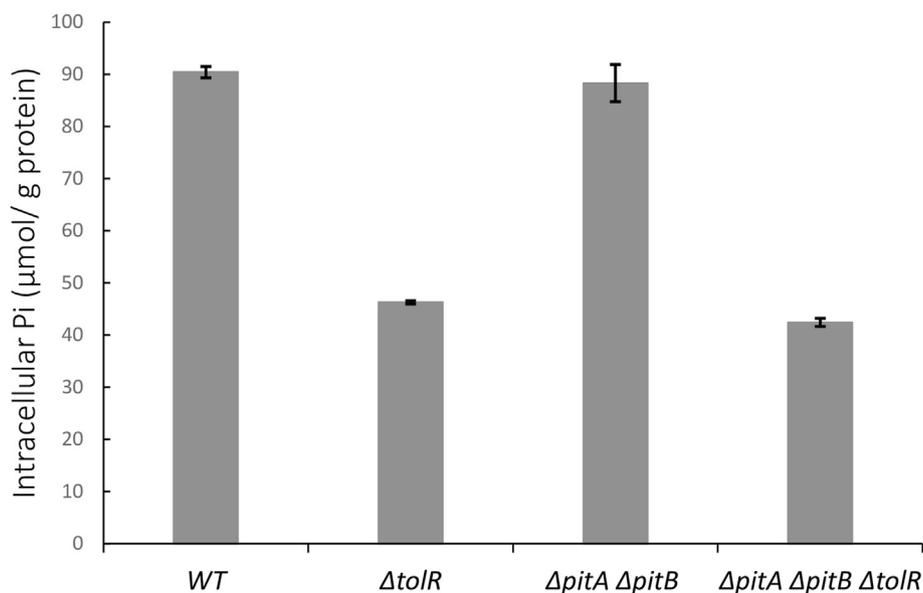


Fig. 6. Intracellular concentration of free Pi in the wild-type, $\Delta tolR$, $\Delta pitA \Delta pitB$ and $\Delta pitA \Delta pitB \Delta tolR$ strains. The intracellular free inorganic phosphate (Pi) concentration was quantified using the BIOMOL GREEN™ reagent for the MG1655 WT strain, the $\Delta tolR$, the $\Delta pitA \Delta pitB$, and the $\Delta pitA \Delta pitB \Delta tolR$ mutant after overnight growth in LB medium. Error bars represent the standard deviation of the mean from three individual experiments conducted in duplicate. The Pi concentration value in the LB medium was 1.7 ± 0.27 mM using the same reagent.

subunit (TolR_{D23C}) result in a TolQRA complex assembled but unable to use PMF. Fig. 8 shows that the intracellular Pi concentration decreased significantly in the $\Delta tolQR$ strain compared to the wild-type and, as expected, complementation occurred when TolQR complex was overproduced in the $\Delta tolQR$ strain. In contrast, complementation was observed neither for TolQR_{D23C} nor for TolQ_{T145A}R overproduction in the $\Delta tolQR$ strain. These data clearly show that energization of the Tol–Pal system is crucial for Pi uptake.

4. Discussion

Our results revealed a contribution of the Tol–Pal system of *E. coli* in the uptake of both oxyanions chromate and phosphate. This contribution could be direct or indirect. Chromate, which is a toxic and non-essential metal for bacteria, is supposed to enter cells by sulfate transporters [1,7]. In this study, we show that chromate uptake requires a functional Tol–Pal system. As expected, when the Tol–Pal system is defective, chromate transport strongly decreases, leading to lower cellular toxicity. The uptake path of chromate has not been clearly established in many strains and, in this context, it is not surprising that an oxyanion importer could allow chromate entrance.

However, a low level of chromate uptake remained in the $\Delta tolQR$ strain showing that another transporter, which could be the *E. coli* sulfate transporter, imports chromate.

The involvement of the Tol–Pal system in Pi import is interesting because this complex has been shown to play a key role in the integrity of the cell envelope and outer membrane homeostasis [35,36]. Mutants of the Tol–Pal system are sensitive to detergents and form filaments in poor media, and group A colicins and filamentous phages benefit from this system to penetrate cells. Recently, the Tol–Pal system has been shown to be pivotal during cell division by enabling outer membrane constriction and septum peptidoglycan synthesis [13,19–21]. Therefore, due to the fact that the Tol–Pal mutants have many phenotypes, Cr(VI) resistance and lower Pi uptake could arise from another function of the system that remains to be determined.

Interestingly, the TonB complex shares homology with the Tol–Pal system [17,18,22,37–39]. The ExbB, ExbD, and TonB inner membrane proteins are homologous to TolQ, TolR, and TolA, respectively. Both systems are energized by PMF to function and interact with their target proteins. The TonB system acts in association with outer membrane transporters to allow the uptake of vital nutrients including heme, iron, and vitamin B₁₂. Similarly, our study suggests that the Tol–Pal system is involved in the uptake of

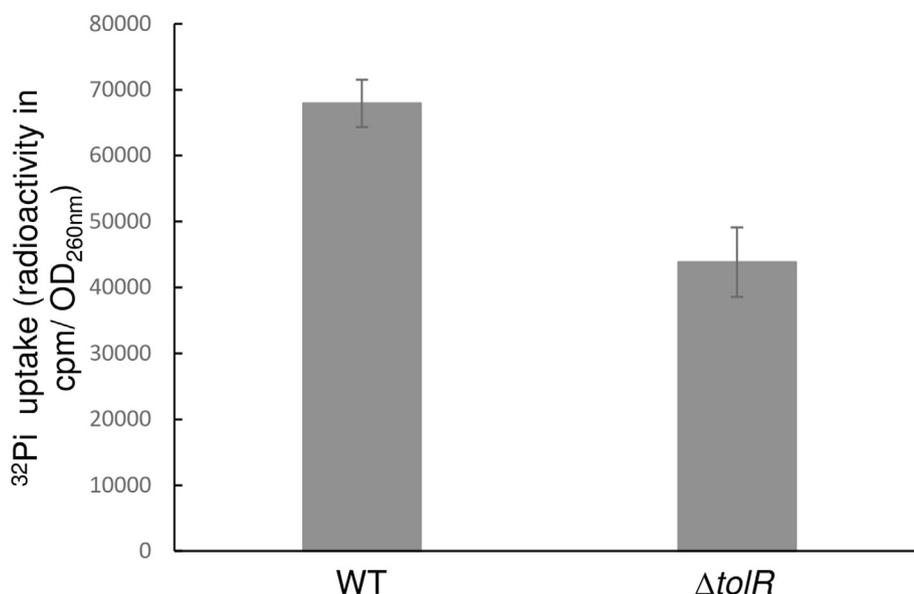


Fig. 7. Level of radiolabeled phosphate (³²Pi) accumulated by the wild-type and $\Delta tolR$ strains. The strains were grown overnight in LB supplemented with 20 μ Ci radiolabeled inorganic phosphate (³²Pi). Uptake was determined as the level of radioactivity (in count per minute, cpm) divided by the sample nucleic acid absorbance at 260 nm, as described in Materials and Methods. Error bars represent the standard deviation of the mean from three individual experiments.

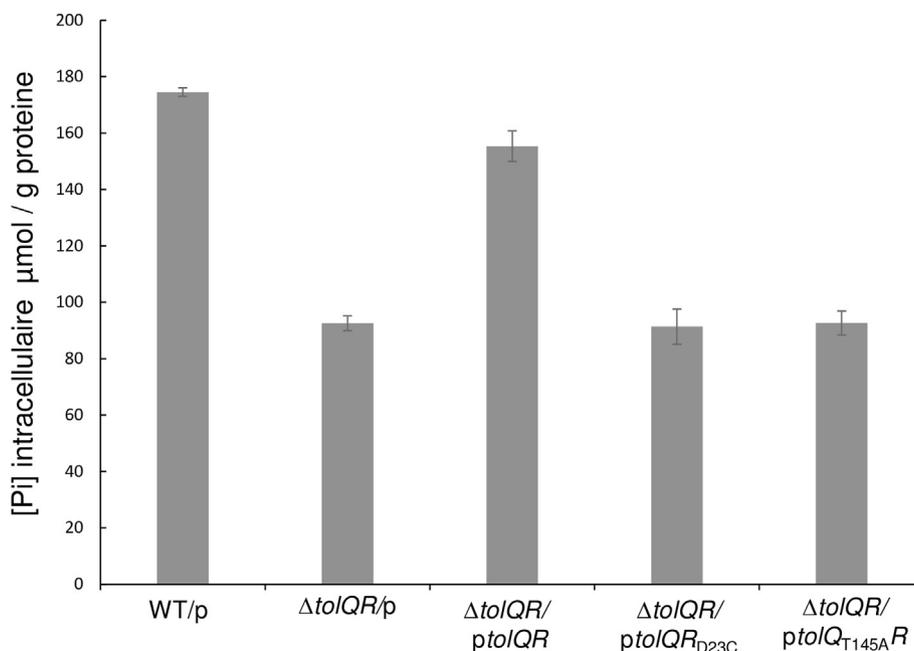


Fig. 8. TolQR function is required for Pi uptake. The intracellular free inorganic phosphate (Pi) concentration was quantified using the BIOMOL GREEN™ reagent for the W3110 WT strain containing pBAD vector (p) and the $\Delta tolQR$ mutant containing either pBAD/HisC vector (p), *ptolQR*, *ptolQR*_{D23C} or *ptolQR*_{T145A} plasmid. The WT and the mutant strains have been grown overnight in LB medium supplemented with 0.002% of L-arabinose. Error bars represent the standard deviation of the mean from three individual experiments conducted in duplicate.

phosphate, an essential molecule. As in the case of the TonB system, the Tol–Pal system might be involved in the transport of other compounds. The Tol–Pal system of *Pseudomonas putida* has been proposed to play some role in the uptake of certain carbon source [40]. Furthermore, given that the Tol–Pal system of *E. coli* is involved in Pi uptake when extracellular phosphate is not a limiting factor, additional experiments are necessary to better understand the effective role of PitA and PitB. Indeed, the $\Delta pitA \Delta pitB$ mutant behaved as the wild-type strain. Interestingly, recent publications

describing a link between Pi uptake and Mg homeostasis in *Salmonella enterica* have revealed the presence of a still unknown Pi transporter [41,42], which might be the Tol–Pal complex of *Salmonella*. However, induction of an oxyanion exporter due to an indirect effect of Tol–Pal mutations remains also a possibility.

One of the most important points is now to understand the potential mechanism of Pi uptake by the Tol–Pal system. The TolQR complex seems essential for the oxyanion transport. The TolQR complex constitutes the inner membrane stator, and energization

of the Tol–Pal system by TolQR is clearly required during import. This means that TolA must cyclic interact with the outer-membrane TolB–Pal complex to promote Pi import. The involvement of an outer-membrane lipoprotein for Pi uptake is puzzling in comparison with the other known uptake systems. We propose that a functional Tol–Pal system could form a trans-envelope scaffold required for efficient Pi import.

In addition, we do not know whether a specific outer-membrane porin is implicated during oxyanion uptake and if the Tol–Pal system operates alone or in concert with another transporter. Chromate toxicity towards *E. coli* will help to decipher the Pi uptake mechanism. Indeed, specific mutants affected in Pi transport could be selected based on chromate resistance phenotype. This simple approach could also be applied to many other bacteria to detect oxyanion importers.

In conclusion, our results open new, unexpected avenues in the field of oxyanion uptake across cell envelopes. They confirm that a well-known system could hide additional direct or indirect physiological roles, but whether the functions performed by the Tol–Pal system are connected or not remains an open question.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Acknowledgments

We thank all members of the group for helpful discussions, especially Sandra Angelini and Olivier Genest. We are grateful to Elsa Germain and Benjamin Ezraty for their kind advices on radioactivity assays. We also thank Baba and colleagues for the Keio mutant collection and Farida Seduk. We also thank Ahmed Zellat for his help on ICP experiments. The manuscript has been professionally proofread ([Proof-Reading-Service.com](https://www.proofreading-service.com)). This research was funded by the Center National de la Recherche Scientifique (www.cnrs.fr) and Aix-Marseille Université (www.univ-amu.fr). D.D and L.H research team is supported by Agence Nationale de la Recherche (MEMOX, ANR-18-CE11).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.resmic.2022.103967>.

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