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Chlorinated phenols control the expression of the multidrug resistance efflux pump MexAB–OprM in *Pseudomonas aeruginosa* by interacting with NalC

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Summary

NalC is a TetR type regulator that represses the multidrug efflux pump MexAB–OprM in *Pseudomonas aeruginosa*. Here we explain the mechanism of NalC-mediated regulation of MexAB–OprM. We show that NalC non-covalently binds chlorinated phenols and chemicals containing chlorophenol side-chains such as triclosan. NalC-chlorinated phenol binding results in its dissociation from promoter DNA and upregulation of NalC's downstream targets, including the MexR antirepressor ArmR. ArmR upregulation and MexR–ArmR complex formation have previously been shown to upregulate MexAB–OprM. *In vivo mexB* and *armR* expression analyses were used to corroborate *in vitro* NalC-chlorinated phenol binding. We also show that the interaction between chlorinated phenols and NalC is reversible, such that removal of these chemicals restored NalC promoter DNA binding. Thus, the NalC-chlorinated phenol interaction is likely a pertinent physiological mechanism that *P. aeruginosa* uses to control expression of the MexAB–OprM efflux pump.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen associated with a wide range of community-acquired and nosocomial infections (Mesaros *et al*., 2007). *P. aeruginosa* infections are responsible for a significant rise in morbidity and mortality in intensive care units (Kerr and Snelling, 2009). Intrinsically resistant to multiple antibiotics, *P. aeruginosa* is a versatile adversary, having the ability to modify and acquire new traits and adapt to diverse environments (Hocquet *et al*., 2007).

Pseudomonas aeruginosa harbours several chromosomal multidrug resistance (MDR) efflux pumps conferring resistance to a variety of antibiotics (Alekshun and Levy, 2007; Lister *et al*., 2009). The MexAB–OprM efflux pump has the widest spectrum among these MDR pumps. It mediates the efflux of diverse antibiotics, such as tetracyclines, fluoroquinolones, β-lactams, chloramphenicol, macrolides, novobiocin, trimethoprim and sulphonamides and biocides such as triclosan (Lister *et al*., 2009). MexAB–OprM also effluxes quorum sensing molecules (Juhas *et al*., 2005) and virulence factors (Piddock, 2006). The apparent lack of specificity of MexAB–OprM is intriguing and its natural physiological role unclear (Neyfakh, 1997; Martinez, 2009).

MexAB–OprM has three known transcriptional regulators: MexR, NalD and NalC (Daigle *et al*., 2007). Among the three regulators, MexR is by far the best studied (Fig. 1A). MexR, which is autoregulated, is transcribed divergently from the same intergenic promoter region as *mexAB–oprM* (Daigle *et al*., 2007) (Fig. 1A). MexR's binding to this intergenic region overlaps with promoters for *mexR* and *mexAB–oprM* and represses their expression. NalD, another repressor, binds a secondary promoter region of MexAB–OprM and downregulates MexAB–OprM expression as well (Morita *et al*., 2006). The autoregulator NalC (Fig. 1A) exerts indirect negative control over MexAB–OprM expression by repressing ArmR, an antirepressor of MexR (Daigle *et al*., 2007). MexR, complexed with ArmR (Fig. 1B), fails to attach to the intergenic promoter region, which results in the overexpression of MexAB–OprM (Wilke *et al*., 2008). Thus, absence of NalC binding to the promoter region will relieve ArmR repression, thereby promoting ArmR's complex formation with MexR and increasing MexAB–OprM expression. In addition to the intricate transcriptional control that MexR expression is subjected to, MexR activity is also regulated on the post-translational level.

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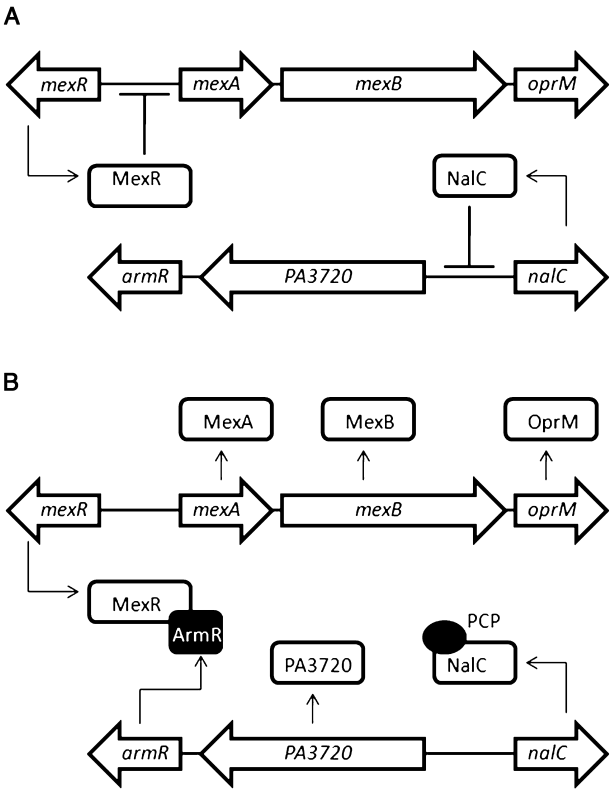


Fig. 1. Regulation of the MexAB–OprM efflux pump in *P. aeruginosa*.
A. PCP not present.
B. PCP present.
⊥ denotes binding to the promoter and inactivation of transcription,
⟶ denotes production of protein.

Oxidation of two cysteines in MexR has been shown to cause conformational changes in the protein, preventing it from binding to the promoter DNA region. This redox-mediated control of MexR function leads to the upregulation of the MexAB–OprM operon during oxidative stress conditions both *in vitro* and *in vivo* (Chen *et al*., 2008).

In this paper we show that chlorinated phenols function as NalC effector molecules by reversibly binding to NalC and decreasing its DNA binding affinity, resulting in de-repression of MexAB–OprM expression (Fig. 1B). NalC is a TetR type regulator with a helix–turn–helix DNA binding domain (DBD) and a ligand binding domain (LBD) (Ramos *et al*., 2005). The interaction between NalC and chlorinated phenols explains the overexpression of MexAB–OprM and upregulation of *mexR*, *nalC* and *armR* observed by Muller *et al*. (2007) in *P. aeruginosa* chemostat cultures in response to treatment with pentachlorophenol (PCP). By using *in vitro* binding studies, we demonstrate that unlike in response to other organic solvents (Li and Poole, 1999), the PCP-mediated upregulation of the *mexAB–oprM* operon is not linked to mutations in MexR but is due to the reversible binding of PCPs to NalC. While chlorinated aromatics including chlorophenols can be produced by natural bacterial and fungal activity (Bengtson *et al*., 2009), chlorophenols are also commercially produced and are widely present in biocides and disinfectants (Weber *et al*., 2007). We address the significance of chlorinated phenol-mediated MexAB–OprM regulation in the light of its environmental occurrences.

Results and discussion

MexAB–OprM regulators are overexpressed in the presence of PCP

In order to corroborate the results of Muller *et al*. (2007), who demonstrated PCP-mediated upregulation of *mexAB–oprM* and its regulators in chemostat cultures, we used quantitative RT-PCR to analyse the expression of *mexB*, *mexR*, *armR* and *nalC* in log phase cultures (OD_{600} ~ 0.3) with and without 120 μM PCP. We observed ~1.5-fold increases in both *mexB* and *mexR* expression in the presence of PCP compared with cultures grown in the absence of PCP (Fig. S1). Expression of *armR*, a direct target of NalC and MexR's antirepressor, increased greater than 100-fold, while *nalC* expression increased threefold to fourfold in the presence of PCP. All increases were statistically significant (P-values < 0.05) based on analysis of variance using MacAnova 5.03. While the fold changes were numerically different, increased expression of these genes in the presence of PCP is consistent with microarray-derived expression data by Muller *et al*. who reported a threefold to fourfold upregulation of *mexAB–oprM* and *mexR* and 9- and 15-fold increases in *armR*, *PA3720* and *nalC* in the presence of ~150 μM PCP.

PCP does not select for mexR *and* nalC *mutants*

Upregulation of the *mexAB–oprM* operon in the presence of organic solvents has been previously shown to be connected to mutations in the *mexR* gene (Li and Poole, 1999). To investigate whether a similar mechanism is responsible for our observed increases in the expression of MexR and NalC-regulated genes, we cultured *P. aeruginosa* PAO1 in the presence of PCP and selected colonies on plates containing 150 μM, 1.5 mM and 3.75 mM PCP. None of the colonies analysed from these PCP plates had mutations in either *mexR* or *nalC*. This result suggests that upregulation of the *mexR* and *nalC* mRNA in the presence of PCP does not require genetic changes in these regulators but functions as a regulated transcriptional response to PCP treatment.

PCP causes dissociation of NalC from its promoter DNA

Upregulation of both *nalC* and *armR* coupled with the lack of detectable mutations in *nalC* led us to hypoth-

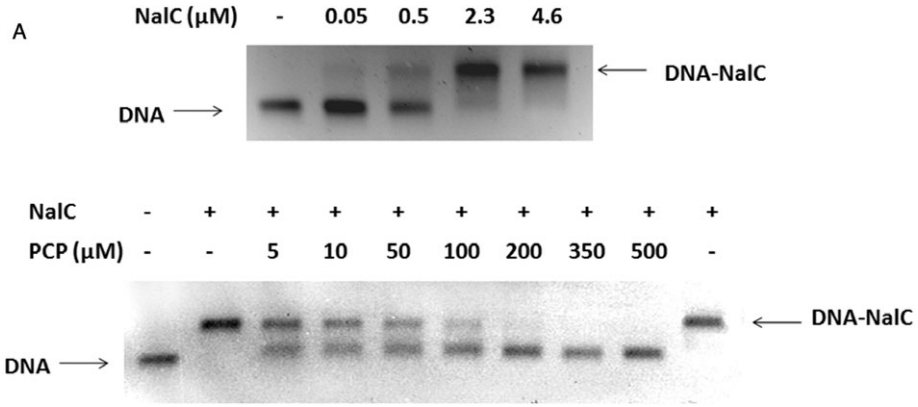


Fig. 2. *In vitro* analysis of interaction between NalC, promoter DNA and PCP.
A. EMSA with 1.2 μM promoter DNA and varying concentrations of NalC.
B. EMSA with 1.2 μM promoter DNA, 4.6 μM NalC and varying concentrations of PCP. Last lane: 70 μM NalC and 7.6 mM PCP incubated followed by PCP removal by gel filtration and incubation with DNA. (+): present, (–): absent.

esize that PCP might be directly binding NalC, thus causing the de-repression of its own expression and that of the downstream target *armR*. ArmR, in turn, would then interact with the repressor MexR, preventing its binding to DNA, and subsequently causing the observed upregulation of *mexR* and *mexAB–oprM*. To test our hypothesis we analysed the binding between NalC and promoter DNA in the presence and absence of PCP using electrophoretic mobility shift assay (EMSA) (Fig. 2A). A 262 bp DNA segment encompassing the *nalC*/*PA3720* intergenic region including the start sites of both these genes and the respective promoters, as identified by Cao *et al*., was used in EMSA (Cao *et al*., 2004). We found that NalC binding to the promoter DNA region reduced the mobility of the 262 bp DNA segment (Fig. 2A). DNA binding was nearly saturated when 2.3 μM NalC and 1.2 μM promoter DNA were combined.

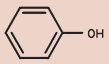
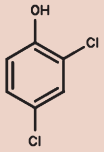
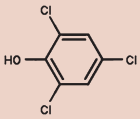
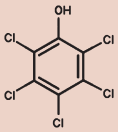
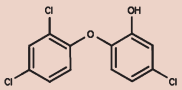
To assess the effects of PCP on NalC's DNA binding affinity, we tested increasing concentrations of PCP in the presence of 4.6 μM NalC and 1.2 μM promoter DNA. As shown in Fig. 2B, the presence of low micromolar concentrations of PCP was sufficient to significantly decrease the binding affinity of NalC to DNA. At concentrations of PCP above 200 μM, no binding of NalC to DNA was observed (Fig. 2B). These results are consistent with the conclusion that PCP acts as a ligand of NalC and prevents NalC from binding DNA.

To exclude the possibility that PCP caused any irreversible modifications of NalC, thus inactivating the DNA binding protein, we incubated 70 μM NalC with 7.6 mM PCP for 1 h. This PCP concentration was sufficient to prevent NalC binding to DNA. Following this incubation, the PCP–NalC mixture was filtered through an Illustra NAP-5 column. The Sephadex gel filtration matrix allows for re-equilibration of protein and ligand and has been used to study reversible protein-ligand binding in the Hummel and Dreyer method (Cann and Hinman, 1976). Following gel filtration, binding between NalC and DNA was completely restored (Fig. 2B, last lane), demonstrating that exposure of NalC to PCP did not permanently change NalC's DNA binding affinity. We concluded from these results that PCP reversibly interacts with NalC to prevent it from binding DNA.

Dichlorophenol, trichlorophenol and triclosan bind NalC

To assess the ligand specificity of NalC, we examined chemicals structurally similar to PCP for their ability to bind NalC and prevent DNA binding. We tested phenol, 2,4-dichlorophenol (DCP) and 2,4,6-trichlorophenol (TCP) and the phenol-based disinfectant triclosan, which has a monochlorophenol group (Table 1). As before, we assessed binding of 4.6 μM NalC to 1.2 μM promoter DNA in the presence of these chemicals. While phenol, even at 20 mM, did not affect NalC–DNA binding (Fig. 3A), we found that both DCP and TCP diminished the apparent binding affinity of NalC to DNA, similar to the results observed with PCP. In contrast to PCP, however, significantly higher concentrations of DCP and TCP were required to prevent NalC binding to DNA. At 1 mM, DCP had minimal effects on NalC's DNA binding while TCP at this concentration partially interfered with NalC–DNA binding. Triclosan also required higher concentrations than PCP to abolish NalC–DNA binding. At 215 μM, triclosan did not affect NalC–DNA binding, while presence of 430 μM triclosan nearly completely prevented NalC's binding to DNA (Fig. 3B). As shown for PCP, DNA binding of NalC was largely restored upon removal of the ligands by dialysis (data not shown). Our

Table 1. Chemicals tested for binding to NalC using EMSA.

Chemical		pK_a	log K_{ow}	Binding to 4.6 μM NalC
Phenol		9.95	1.46	No binding with 20 mM
2,4-DCP		7.8	3.06	Yes (complete at 9 mM)
2,4,6-TCP		6.0	3.72	Yes (partial at 2.5 mM)
PCP		4.74	5.12	Yes (complete at 125 μM)
Triclosan		7.9	4.76	Yes (partial at 430 μM)

pK_a and log K_{ow} values are from ChemIDplus (United States National Library of Medicine).
K_{ow}, octanol-water partition coefficient. It is the ratio of the concentration of the chemical in octanol and in water. Octanol is used as a surrogate for natural organic matter.

findings that the chlorinated phenol derivatives prevent NalC's binding to the promoter region *in vitro* were in excellent agreement with *in vivo* expression studies of *armR* and *mexB* (Fig. 3C). We found that *armR* and *mexB* transcription levels were reproducibly increased in the presence of DCP, TCP and triclosan, but not in the presence of phenol. These results strongly suggest that chlorination of the phenol is an important characteristic of NalC ligands.

Chlorophenols are weak acids. While PCP shows a pK_a of 4.74 indicating that it is almost completely de-protonated under our assay conditions (pH 7.8), DCP shows a pK_a of 7.8 and is predicted to be about 50% de-protonated. We were curious about the relative strength of the protonated versus the de-protonated forms in interacting with NalC and abolishing DNA binding, which might explain the observed differences in relative affinity of the ligands. We found that binding of NalC to DNA was relatively unaffected in the pH range of 6–9 (Fig. 3D). Similarly, the effects of PCP on NalC's binding affinity were pH-independent in the chosen pH range where PCP is largely de-protonated. In stark contrast, however, DCP at pH below 8 (primarily protonated) completely prevented NalC–DNA binding (Fig. 3D). NalC–DNA binding increased in the presence of DCP with increasing pH values, indicating that the protonated form has higher affinity for NalC. These results suggest that the protonation state of the phenol alone cannot explain NalC binding affinities.

Thermodynamic stability of NalC

The regulatory mechanism of most TetR family members involves the binding of ligands to their LBD, which in turn induces the dissociation of DNA from their DBD. A widely accepted explanation for TetR's allosteric mechanism is that the protein assumes two distinct structures in the ligand-free and ligand-bound states, one that binds DNA and the other that does not (Orth *et al*., 2000; Ramos *et al*., 2005). Recently, a different mechanism was proposed by Reichheld *et al*. (2009), who suggested that the ligand-free TetR has a flexible DBD, arising from a lack of interaction between the DBD and the LBD, and that this flexibility of the DBD is important for DNA binding. The authors then showed that the ligand-free TetR unfolds in a three-state manner, with the unfolding of the DBD preceding the unfolding of the LBD. Binding to the ligand tetracycline increased cooperativity between the two domains of TetR, resulting in rigidification of the DBD and a single cooperative unfolding transition. Based on these data, Reichheld *et al*. suggested that lack of flexibility of the DBD in the ligand-bound state is responsible for its failure to bind DNA.

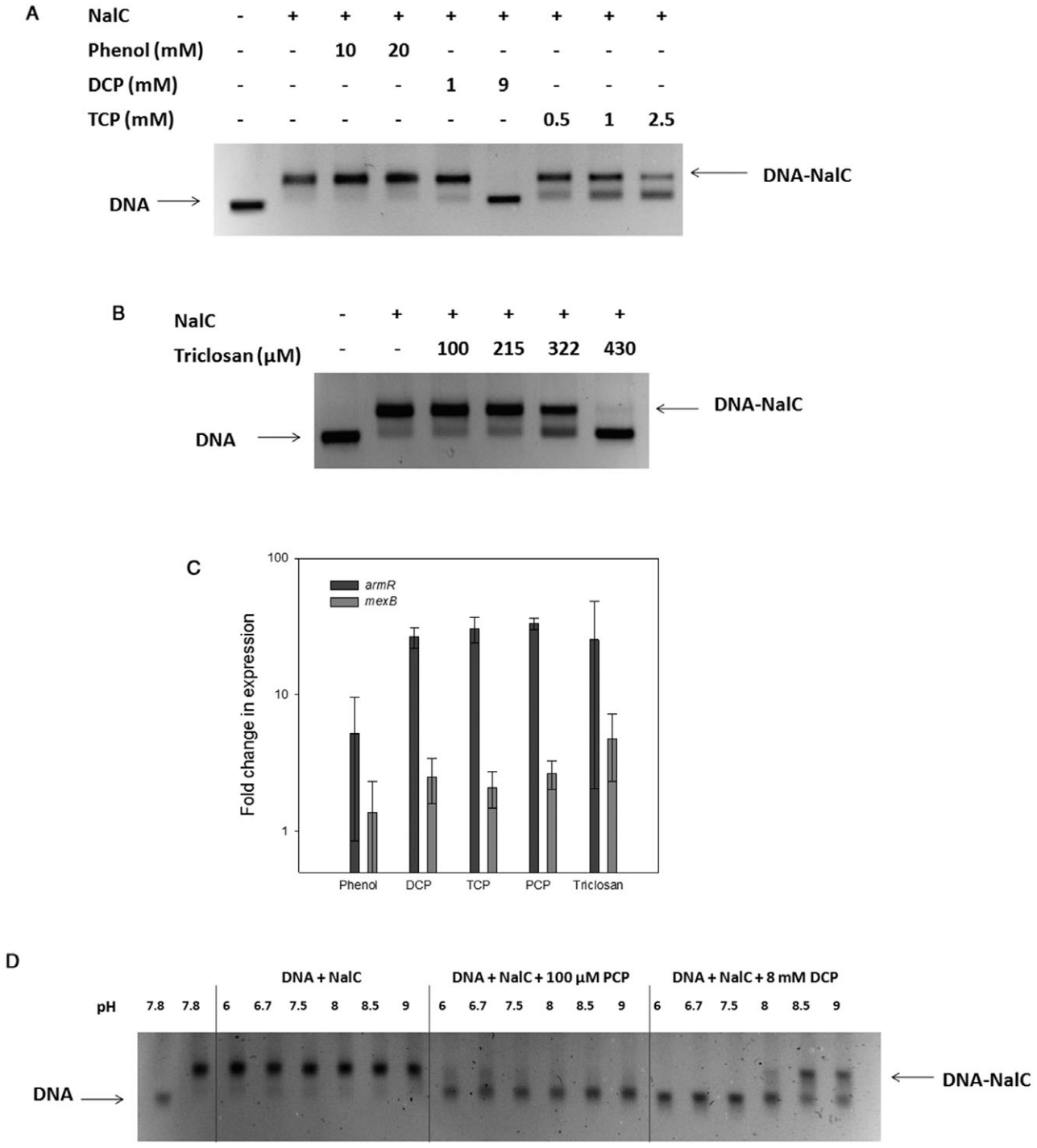


Fig. 3. Interaction of phenolics with NalC.
A. EMSA with 1.2 μM promoter DNA, 4.6 μM NalC and varying concentrations of phenol, DCP and TCP.
B. EMSA with 1.2 μM promoter DNA, 4.6 μM NalC and varying concentrations of triclosan.
C. Fold change in expression of *armR* and *mexB* normalized to *rpsL* after addition of 5 mM phenol, 300 μM DCP, 400 μM TCP, 120 μM PCP or 100 μM triclosan to log phase batch cultures. Bars represent means from three separate batch cultures and error bars represent standard deviations about the mean.
D. EMSA with 1.2 μM promoter DNA and 4.6 μM NalC. PCP and DCP were used as indicated. pH was varied between 6 and 9.
(+): present, (–): absent.

To monitor the conformational rearrangements of NalC upon ligand binding and its stability in the absence and presence of PCP, we conducted Far-UV circular dichroism (CD) measurements of NalC as read-out for changes in its secondary structure. We found that in the presence of PCP, NalC gained helicity, suggesting significant structural rearrangements in the LBD upon PCP binding (Fig. 4A). Thermal transition data fitted with sigmoidal curves in SigmaPlot 10 show that the apparent median melting temperature of NalC also increased from 59°C in the absence of PCP to 78°C in the presence of PCP, confirming that PCP binds to NalC, hence the increased stability (Fig. 4B). Upon removal of PCP, NalC behaved like the ligand-free protein, providing further support for the reversibility of NalC–PCP binding. Incubation of NalC in presence of 1.25 mM phenol, a ligand that does not bind NalC, did not affect the structure of NalC (Fig. S2). To further exclude that non-specific effects of PCP might be responsible for the observed changes in NalC's structure and thermal stability, we analysed the CD spectrum and thermal transition of the redox-sensitive chaperone Hsp33 in the presence of 400 μM PCP (Fig. S3). Hsp33 is a two-domain protein containing a meta-stable linker region, and is not known to have ligand binding properties (Graf *et al.*, 2004). As shown in Fig. S3, we were unable to detect any effect of PCP on the structure or thermal stability of Hsp33. These results strongly suggest that the binding of PCP to NalC is specific and increases the stability of the regulator. In contrast to the reported results on TetR, however, ligand-free NalC showed a classical two-state transition whereas the unfolding of PCP-bound NalC was non-cooperative (Fig. 4B), suggesting that the effects of ligand binding might differ among the members of this large protein family. However, neither one of the transitions was fully reversible, precluding us from precisely assessing the role of ligand binding on the stability of NalC.

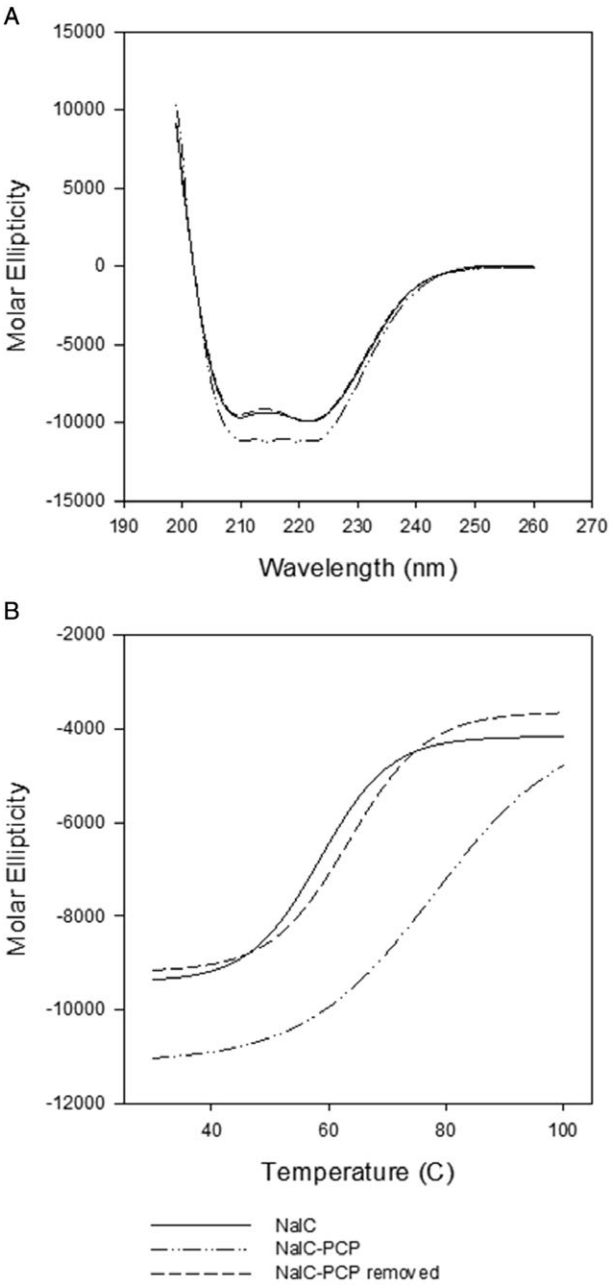


Fig. 4. Effect of PCP on the thermodynamic stability of NalC.
A. CD spectra of NalC (straight line), NalC bound to PCP (dashed and dotted line) and NalC incubated with PCP followed by PCP removal by gel filtration (dashed line).
B. Thermal transitions curves for NalC (straight line), NalC bound to PCP (dashed and dotted line) and NalC incubated with PCP followed by PCP removal by gel filtration (dashed line).
The samples were heated with a rate of 1°C min^{-1} and the CD signal at 222 nm was monitored. The thermal transitions are not completely reversible.

Significance of NalC activation by chlorinated phenols

Our observation that NalC expression is induced by chlorinated phenols leads us to speculate on its natural physiological significance. Halogenated organics are naturally present in soil (Myneni, 2002). Different chlorinated phenols, particularly chlorinated methoxyphenols, have been detected in pristine river waters in the ppb (μg l^{-1}) concentration range (Michalowicz *et al.*, 2008). We looked at *armR* expression in the presence of varying concentrations of PCP, and found that concentrations as low as 40 ppb of PCP significantly increased levels of expression of *armR* in *P. aeruginosa* (data not shown). Many soil microorganisms, including bacteria and fungi, harbour halogenases and haloperoxidases and potentially generate organohalogens (Bengtson *et al.*, 2009; Wagner *et al.*, 2009). These observations suggest that *P. aeruginosa*, which is also found in soil (Schobert and Tielen, 2010), is likely to encounter chlorinated organics including chlorophenols in its natural habitat and may have evolved

NalC-mediated efflux to protect itself. While addition of 150 μM PCP to logarithmic phase batch cultures of *P. aeruginosa* PAO1 did not affect its growth rate (Fig. S4A), strains lacking the functional *mexB* or *armR* gene showed reduced growth in the presence of PCP (Fig. S4B and C). Curiously enough, *P. aeruginosa* also harbours a functional chloroperoxidase (Song *et al*., 2006).

Apart from naturally produced chlorinated phenols, *P. aeruginosa* is likely to be exposed to these chemicals also because of human activities. Chlorophenols, such as 2-chlorophenol, 2,4-DCP, 2,4,6-TCP and PCP have long been used as biocides and wood preservatives and can contaminate soil (McLellan *et al*., 2007). Chlorination of drinking water also results in the production of minor quantities of chlorophenols (Ge *et al*., 2008). Both are environments that harbour *P. aeruginosa* (Mena and Gerba, 2009; Schobert and Tielen, 2010). Another environment where *P. aeruginosa* may be exposed to chlorophenols is health-care units (Weber *et al*., 2007). Triclosan is an antimicrobial used both in health-care units and in a wide variety of household products (Fiss *et al*., 2007). Other examples of chlorophenol-based disinfectants include chloroxylenol and ortho-benzyl-para-chlorophenol (Rutala *et al*., 2008). The effect of low doses of different halogenated phenols in these environments on *P. aeruginosa* survival, proliferation and other characteristics such as resistance and virulence remains a pertinent question.

Conclusion

We show here that chlorinated phenols interact with the transcriptional regulator NalC of the MexAB–OprM MDR efflux pump to control its expression. NalC binding to chlorinated phenols results in de-repression of NalC and ArmR. Increased expression of ArmR, a MexR antirepressor, results in MexR–ArmR complex formation (Wilke *et al*., 2008) and alleviates MexR-mediated repression of MexAB–OprM. We demonstrated that NalC-chlorinated phenol binding is fully reversible as NalC regained its DNA binding activity once chlorophenols were removed. We found that triclosan also reversibly binds to NalC. This observation expands the range of chemicals that potentially induce NalC to include chemicals with chlorophenol side-chains. It will now be interesting to determine the precise ligand recognition mechanism that is used by NalC.

As we continue to use and release various purportedly toxic or benign chemicals, it is important for us to understand their full potential in impacting the biosphere. Now we know that antibiotics do not only have antagonistic roles (Linares *et al*., 2006) and that mechanisms of resistance to antibiotics did not necessarily evolve in response to antibiotics (Piddock, 2006). Similarly, transcriptional regulators are not simple on/off switches, and responses are modulated by the intensity of signalling molecules as well as the different regulatory pathways that intersect to produce a functional organism (Cases and de Lorenzo, 2005). Our observation that chlorinated phenols induce a MDR efflux pump regulator fits these paradigms.

Experimental procedures

Bacterial strains and growth conditions

Wild-type *P. aeruginosa* strain PAO1 was obtained from H. Schweizer (Schweizer, 1998). PAO1 was grown in Luria–Bertani (LB) medium at 37°C unless otherwise stated. *Escherichia coli* BL21(DE3) [B834 derivative, F $^{-}$*ompThsdS*$_B$($r_B^- m_B^-$) *gal dcm* (DE3), Novagen, EMD Biosciences, San Diego, CA, USA] with the pet-15b plasmid (expression vector with N-terminal His • Tag® Ap^r, Novagen) was grown on LB with 50 μg ml^{-1} of ampicillin at 37°C.

Chemicals

Pentachlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and triclosan were purchased from Sigma-Aldrich (Table 1). PCP stock was made at 10 mg ml^{-1} in 36 mM NaOH. DCP stock was made at a concentration of 5 mg ml^{-1} in Tris buffer (pH 7.8). TCP stock was made at 5 mg ml^{-1} in 36 mM NaOH. Triclosan stock was made at 1.25 mg ml^{-1} in 50% solution of 36 mM NaOH. Concentrations of DCP and TCP used in experiments were verified using HPLC.

Gene expression analysis using qRT-PCR

Pseudomonas aeruginosa PAO1 was grown in minimal medium (Muller *et al*., 2007) with and without 120 μM PCP. Triplicate batch cultures were used for both growth conditions. Samples were collected in duplicate during logarithmic growth phase (OD_{600} ~ 0.3), immediately treated with RNAprotect Bacterial Reagent (Qiagen, Valencia, CA, USA) and stored at –80°C for RNA extraction. Gene expression was also analysed in the presence of 300 μM DCP, 400 μM TCP and 100 μM triclosan. PCP (120 μM) and phenol (5 mM) were used as positive and negative control respectively. Each condition was tested in triplicate. For this experiment *P. aeruginosa* PAO1 was grown in LB to early logarithmic growth phase (OD_{600} ~ 0.2), when chemicals were added. Cells were harvested at OD_{600} ~ 0.6 (still in logarithmic growth phase), immediately treated with RNAprotect Bacteria Reagent and stored at –80°C. RNA was extracted using the RNeasy Mini Kit (Qiagen). DNase I, Amplification Grade (Invitrogen Life Science, Carlsbad, CA, USA) was used for DNase digestion prior to cDNA synthesis using the SuperScript II RT kit (Invitrogen Life Science). Random hexamer primers were used for cDNA synthesis. Multiple aliquots of cDNA were stored at –20°C for RT-PCR analysis.

Primers used in qRT-PCR for *mexR*, *mexB*, *armR* and *nalC* genes are listed in Table 2. Housekeeping genes, *nadB* and *rpsL* were used as controls. Quantitative PCR was performed

Table 2. Primers for RT-qPCR.

Name	Sequence (5′ → 3′)	Reference
nalC	F: CCT CAC ATG GAC GAG GAA AC R: AGG TAG CAG GCG ATG ATG TC	This study
armR	F: CCT GAA CAC TCC GCG CAA C R: GTG CTC GCC GTA GAG GTC C	Cao *et al.* (2004)
mexR	F: GAG CTG GAG GGA AGA AAC CT R: AGG CAC TGG TCG AGG AGA T	This study
mexB	F: GTG TTC GGC TCG CAG TAC TC R: AAC CGT CGG GAT TGA CCT TG	Muller *et al.* (2007)
nadB	F: CTTCACCGTGGAGCATAGC R: GCCTTCCTCGTGGTTGTG	Muller *et al.* (2007)
rpsL	F: TAC ATC GGT GGT GAA GGT CA R: TAC TTC GAA CGA CCC TGC TT	This study

in a Mastercycler ep *realplex* thermocycler (Eppendorf, Hauppauge, NY, USA) using the Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). PCR conditions and primer concentrations were optimized to eliminate the formation of primer-dimers and non-specific products. All qPCR analyses were performed in triplicate.

Selection of colonies with high PCP tolerance

To test if PCP selected for *P. aeruginosa* PAO1 with mutations in regulatory genes, colonies grown in medium containing PCP were screened for mutations. Briefly, cultures were grown overnight with 120 μM PCP in LB broth, transferred into fresh PCP-containing LB broth and grown to mid-log phase. Logarithmic phase cultures were 10-fold serially diluted in 10 mM phosphate buffer saline (pH ~ 7.5) and 100 μl cultures were plated onto LB agar containing PCP at concentrations of 150 μM, 1.5 mM, 3.75 mM and 6 mM. No growth was observed at 6 mM PCP. Colonies were randomly picked from plates containing 150 μM, 1.5 mM and 3.75 mM PCP (10 colonies per PCP concentration) and analysed for mutations in *mexR* and *nalC*. Colony PCR was used for amplification of the entire length of these genes. Primers used for amplifying *mexR* were forward 5′-CATTAGGTT TACTCGGCCAAACC-3′ and reverse 5′-CGCCAGTAAGC GGATACCTG-3′ (Daigle *et al.*, 2007) and *nalC* were forward 5′-GAATGAAGCGGAAGTGCTTGC-3′ and reverse 5′-CGA GATCCACCTCACCGAAC-3′(Cao *et al.*, 2004). Amplicons were sequenced at the DNA Sequencing Core facility at the University of Michigan (Ann Arbor).

Expression and purification of NalC

Wild-type *nalC* was PCR amplified from *P. aeruginosa* PAO1 using forward primer 5′-GTGTGTAAGGCATATGAACGATGC TTCTCC-3′ (NdeI site underlined) and reverse primer 5′-T TCGTATTGGATCCACCTCACCGAACTGC-3′(BamHI site underlined), cloned into pET-15b vector containing the 6 × His tag (EMD Biosciences) and transformed into *E. coli* BL21(DE3) (EMD Biosciences). BL21(DE3) with *nalC* was grown to OD_{600} of ~0.6 and induced with 1 mM IPTG for 3 h. Cells were pelleted, resuspended in phosphate buffer (40 mM KH_2PO_4, 200 mM KCl, pH 7.5) and treated with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL, USA). Cells were lysed in the French press. 6 × His-NalC was purified using a bench-top process using the HisPur Cobalt Resin (Thermo Fisher Scientific) following the manufacturer's protocol. NalC was eluted with a 100 mM imidazole buffer (50 mM KH_2PO_4, 300 mM NaCl, pH 7.4). Purity of 6 × His-NalC was assessed using SDS-PAGE analysis on a 14% Tris-glycine gel (Invitrogen). 6 × His tag removal was performed using Restriction Grade Thrombin (EMD Chemicals) followed by elution through the HisPur Cobalt Resin. The cleaved protein was concentrated and loaded onto a HiLoad 26/60 Superdex 75 Prep grade column (GE Healthcare, Piscataway, NJ, USA) equilibrated with HEPES buffer (40 mM HEPES, 100 mM KCl, pH 7.8). Peak fractions were analysed for the presence of pure NalC using SDS-PAGE. NalC-containing fractions were pooled and concentrated to 2 mg ml^{-1}. Protein purity was > 97% as assessed by SDS-PAGE analysis.

Electrophoretic mobility shift assay

The *nalC*/*PA3720* intergenic promoter region (Cao *et al.*, 2004) was amplified using primers 5′-AGGCATCGATAT CCAACAGG-3′ and 5′-GGGAGAAGCATCGTTCAT-3′ and amplification products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). EMSA was set up with purified NalC, promoter DNA and varying concentrations of chemicals in a 10 mM Tris-HCl binding buffer (pH 7.8) containing 1 mM EDTA, 100 mM KCl, 5% v/v glycerol, 0.1 mM DTT, 0.01 mg ml^{-1} BSA (Hellman and Fried, 2007). The NalC protein was pre-incubated with the respective chemicals in the 10 mM Tris-HCl buffer for 1 h. Then, DNA was added and incubation was continued for another 45 min. Samples were run on 0.8% agarose gels in 1 × TAE at 100 V at room temperature and stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA). DNA bands were visualized using a Dark Reader Transilluminator (Clare Chemical Research).

NAP-5 filtration and dialysis

PCP–NalC mixtures were filtered through an Illustra NAP-5 column (GE Healthcare Lifesciences, Piscataway, NJ, USA) packed with Sephadex™ G-25 and equilibrated with

PCP-free 10 mM Tris-HCl binding buffer to remove any non-covalently bound PCP. Similarly, DCP, TCP or triclosan were removed from NalC by dialysis using a regenerated cellulose Spectra/Por membrane with an 8 kDa cut-off (Spectrum Laboratories). TCP and triclosan were dialysed overnight, while DCP was dialysed for 2 days.

Circular dichroism spectroscopy

NalC was diluted to 8.7 μM (0.2 mg ml^{-1}) in 20 mM KH_2PO_4 buffer (pH 7.8). For monitoring the molar ellipticity of NalC bound to PCP, 8.7 μM NalC was pre-incubated with 400 μM PCP for 1 h at room temperature. This PCP concentration was sufficient to completely prevent NalC's binding to DNA. To assess reversibility of PCP binding, NalC–PCP was gel filtered through an Illustra NAP-5 column as described previously and retested. Far-UV CD scans (199–260 nm) for NalC, NalC–PCP and NalC after PCP removal were performed using a Jasco J-810 CD spectrophotometer (Jasco Analytical Instruments, Easton, MD, USA). Six scans were accumulated. The spectra of buffer alone or buffer with PCP were subtracted from the protein spectra. Thermal transitions of NalC, NalC–PCP and NalC after PCP removal were analysed between 30°C and 100°C (temperature was controlled by a Jasco PTC-423S) and readings were taken at 222 nm. The rate of temperature increase was 1°C min^{-1}. Thermal transition data were fitted with sigmoidal curves in SigmaPlot 10 to determine apparent median melting temperatures.

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