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A NarX–Tar chimera mediates repellent chemotaxis to nitrate and nitrite

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Summary

Membrane receptors communicate between the external world and the cell interior. In bacteria, these receptors include the transmembrane sensor kinases, which control gene expression via their cognate response regulators, and chemoreceptors, which control the direction of flagellar rotation via the CheA kinase and CheY response regulator. Here, we show that a chimeric protein that joins the ligand-binding, transmembrane and linker domains of the NarX sensor kinase to the signalling and adaptation domains of the Tar chemoreceptor of *Escherichia coli* mediates repellent responses to nitrate and nitrite. Nitrate induces a stronger response than nitrite and is effective at lower concentrations, mirroring the relative sensitivity to these ligands exhibited by NarX itself. We conclude that the NarX–Tar hybrid functions as a bona fide chemoreceptor whose activity can be predicted from its component parts. This observation implies that ligand-dependent activation of a sensor kinase and repellent-initiated activation of receptor-coupled CheA kinase involve a similar transmembrane signal.

Introduction

The Tar protein of *Escherichia coli* mediates attractant responses to aspartate and maltose (Springer *et al*., 1977) and repellent responses to Ni^{2+} and Co^{2+} ions (Tso and Adler, 1974). It is one of two high-abundance chemoreceptors in *E. coli* (Koman *et al*., 1979). The other is Tsr, which mediates attractant responses to serine and repellent responses to leucine. The crystallographic structures of the periplasmic domain of Tar (Milburn *et al*., 1991; Bowie *et al*., 1995) and the cytoplasmic domain of Tsr (Kim *et al*., 1999) show that these proteins form homodimers in the presence or absence of ligands. The osmosensing EnvZ kinase of *E. coli* is also a homodimer (Yang and Inouye, 1991), and it has a predicted membrane topology similar to that of the chemoreceptors (Forst *et al*., 1987).

Each subunit of Tar spans the membrane twice and extends an N-terminal ligand recognition loop into the periplasmic space and a C-terminal signalling and adaptation domain into the cytoplasm (Krikos *et al*., 1983). The periplasmic domain is responsible for the interaction with most attractant and repellent ligands, including aspartate, maltose-binding protein (MBP) and Ni^{2+} for Tar and serine and leucine for Tsr (Krikos *et al*., 1985). Aspartate binds at the dimer interface of Tar near the apex of the periplasmic domains (Milburn *et al*., 1991). Ligand-bound MBP in a closed conformation (Spurlino *et al*., 1991) is predicted to bind in quasi-symmetrical fashion at the apex of the Tar dimer, with the N-terminal domain in contact with one Tar subunit and the C-terminal domain in contact with the second subunit (Zhang *et al*., 1999).

A number of functional chimeric chemoreceptors have been constructed. The fusion joint for these constructs is in the cytoplasmic domain near the C-terminal end of the linker region (Kalman and Gunsalus, 1990; Williams and Stewart, 1999) that connects the second transmembrane helix (TM2) to the signalling and adaptation domains. This position corresponds to a conserved *Nde*I restriction site present in the *tar* and *tsr* genes of *E. coli* (Tsr is the serine chemoreceptor.) This site spans a CATATG sequence that encodes adjacent His and Met residues. The Tasr and Tsar hybrids, made by reciprocal exchange of the coding regions on either side of the *Nde*I site, have the ligand-sensing properties that correspond to the periplasmic domain of the hybrid (Krikos *et al*., 1985). Subsequent studies have shown that *Nde*I sites introduced at the same relative positions in the *trg* and *tap* genes can be used to generate functional chimeras between the Trg (ribose/galactose) receptor and Tsr (Feng *et al*., 1998) and between the Tap (dipeptide) receptor and Tar (Weerasuriya *et al*., 1998).

To test whether a similar architecture dictates a similar mechanism of transmembrane signalling, chimeras have been constructed between Tar and EnvZ (Utsumi *et al*., 1989) and between Trg and EnvZ (Baumgartner *et al*., 1994). An *Nde*I site was generated in *envZ* at a location judged to be comparable with that of the *Nde*I site in

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tar and was used to join the sensing domain of Tar or Trg to the signalling domain of EnvZ. The level of β-galactosidase expressed from an *ompC–lacZ* fusion gene was used to monitor the activity of the resulting Taz and Trz hybrids. (Transcription of *ompC* requires a high concentration of the phosphorylated form of OmpR, the cognate response regulator for EnvZ.) The addition of aspartate to cells expressing Taz, or of ribose or galactose to cells expressing Trz, led to a substantial increase in β-galactosidase activity. However, the addition of maltose to cells producing Taz did not boost the expression of *ompC–lacZ* (Utsumi *et al.*, 1989).

One problem with using EnvZ to create chemoreceptor–sensor kinase hybrids is that the function of the reciprocal chimera cannot be tested, because EnvZ lacks a known ligand. Deletion analysis indicates that the periplasmic domain of EnvZ is largely dispensable for osmosensing. It can even be exchanged with the periplasmic domain of a non-homologous sensor kinase, PhoR of *Bacillus subtilis*, without impairing osmoregulation (Leonardo and Forst, 1996). Without knowing how the reciprocal hybrids function, it is impossible to evaluate fully how similar the process of transmembrane signalling is in sensor kinases and chemoreceptors.

We thus decided to construct hybrids between the NarX sensor kinase of *E. coli* (Stewart and Berg, 1988; Kalman and Gunsalus, 1990) and Tar. NarX regulates the expression of genes whose products (e.g. nitrate or nitrite

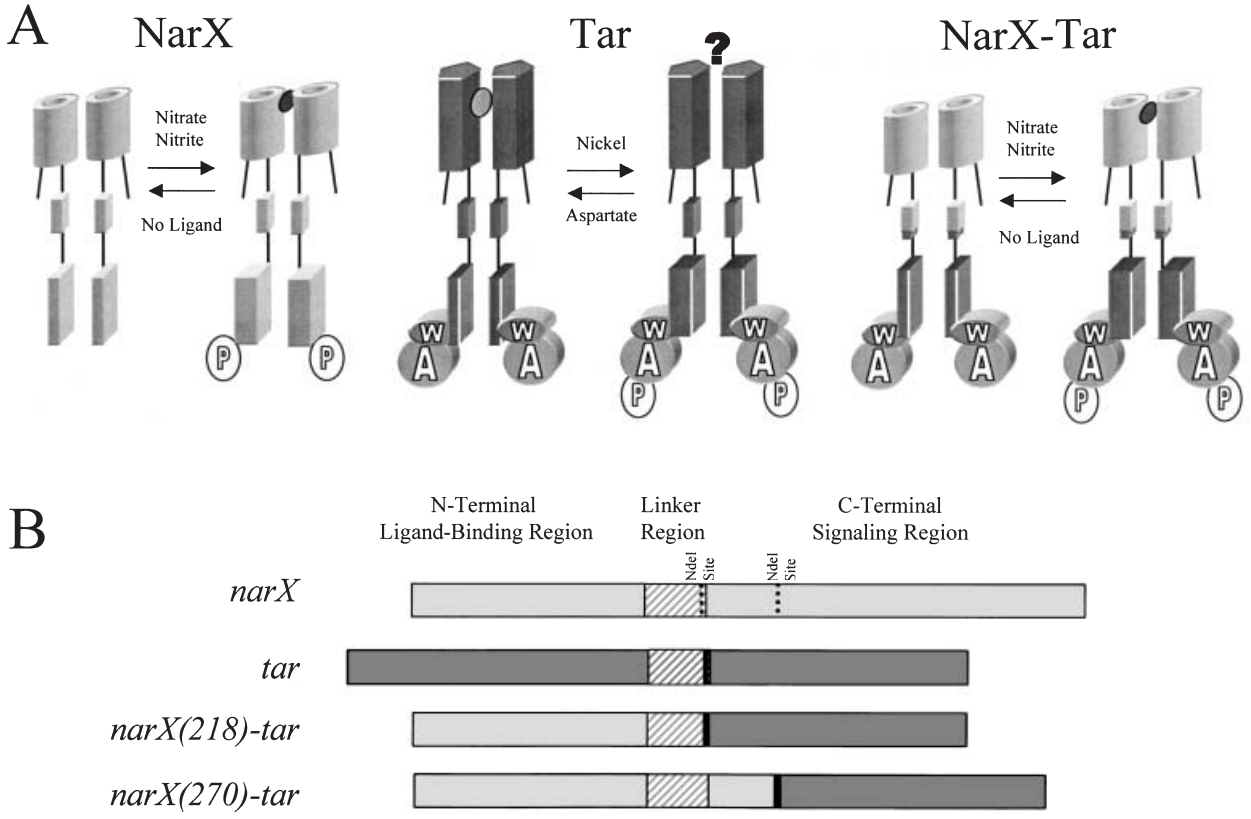

Fig. 1. NarX–Tar chimera function and construction.
A. Under anaerobic conditions, the NarX sensor kinase of *E. coli* mediates a response to nitrate and nitrite (small black oval) in the environment by increasing its rate of autophosphorylation. The phosphoryl group is then transferred to either the NarL or the NarP response regulator, which in the phosphorylated form activates the transcription of genes whose products are involved in anaerobic respiration. The Tar receptor binds the attractant ligands aspartate (larger grey oval) and maltose-bound maltose-binding protein and, in an unknown fashion, the repellent ligands Ni^{2+} and Co^{2+} (question mark). Attractants inhibit the ability of Tar to stimulate CheA autophosphorylation, thereby decreasing phosphotransfer to the CheY response regulator. Repellents enhance the ability of Tar to stimulate CheA autophosphorylation. As *CheY-P* increases the probability of clockwise (tumbling) flagellar rotation, attractants promote smooth swimming and repellents promote tumbling. The NarX–Tar hybrid contains the periplasmic ligand-binding region and the transmembrane and linker domains of NarX and the cytoplasmic signalling and adaptation regions of Tar. A scenario in which nitrate and nitrite stimulate autophosphorylation of CheA is shown.
B. *Nde*I sites were introduced at two positions in the *narX* gene to permit fusion of the N-terminal portion of NarX to the C-terminal portion of Tar. (The *tar* gene has a naturally occurring *Nde*I site at the end of the sequence encoding the linker domain.) The position of the *Nde*I site in the *tar* and *narX–tar* genes is shown as a vertical bar, and the positions at which *Nde*I sites were introduced into *narX* are shown as dotted lines. The narrow vertical lines show the boundaries between sequences encoding the transmembrane/periplasmic and linker regions and the linker and signalling domains, respectively, in each gene. Fusions were made after codons 218 and 270 of *narX*. Only the product of the shorter *narX–tar* fusion gene is stable.

reductase) are involved in the utilization of nitrate or nitrite as terminal electron receptors under anaerobic conditions (Rabin and Stewart, 1993). These ligands bind to the periplasmic domain of NarX (Cavicchioli *et al*., 1996; Williams and Stewart, 1997; Lee *et al*., 1999) and thereby increase its autophosphorylation activity. The phosphoryl group is transferred to either of two response regulators, NarL or NarP, which act as positive or negative transcription factors for a number of genes (Stewart, 1993).

We report here that a NarX–Tar chimera (Nart) can serve as a repellent chemoreceptor for nitrate and nitrite. The active fusion was made using an *Nde*I site introduced into *narX* at the same relative position as that introduced into *envZ* to make Taz and the natural *Nde*I site in *tar*. The chemotactic behaviour of cells producing the chimeric receptor suggests that ligands stimulate the kinase activity of CheA associated with Nart. We infer that the two responses are elicited by a similar transmembrane signal.

Results

The pAD56 plasmid produces a stable NarX–Tar hybrid protein

Two *narX–tar* fusion genes were constructed using *Nde*I restriction sites introduced at two places in *narX* (Fig. 1). Antibody directed against the conserved cytoplasmic domain of Tsr (Ames and Parkinson, 1994) was used as a probe for the hybrid proteins on immunoblots. Plasmid pAD56, in which codons 1–218 of *narX* are joined to codons 257–553 of *tar* (*narX(218)–tar*), produced a cross-reacting protein (Nart) of the expected size (≈ 55 kDa) in the transducer-deleted (ΔT) strain VB13 (Fig. 2). The fusion joint in this hybrid was at the end of the predicted linker domain (Williams and Stewart, 1999). No cross-reacting protein was seen in extracts from strain VB13 containing plasmid pAD48, in which codons 1–269 of *narX* were joined to codons 257–553 of *tar*. The Nart hybrid protein was found in significantly higher amounts in cells that make Tsr and Trg (Δ*tar–tap* strain MM509) than in cells from strain VB13 (Fig. 2). We presume that the presence of Tsr and/or Trg stabilizes the fusion protein.

Several other reproducible features of the immunoblots should be mentioned. First, cells of the wild-type strain RP437 contained about twice as much Tsr as cells of strain MM509, although these strains are isogenic except for the *tar–tap* deletion in MM509. The presence of plasmid-encoded Tar or Nart in strains MM509/pMK113 and MM509/pAD56 did not restore the level of Tsr seen in strain RP437. Secondly, although the level of Nart encoded by plasmid pAD56 was much reduced in strain VB13 relative to strain MM509, the level of plasmid-encoded Tar was approximately the same in strains VB13/pMK113 and MM509/pMK113. We presume that interactions among the receptors and their attendant Che proteins with the receptor patch (Maddock and Shapiro, 1993; Shimizu *et al*., 2000) can lead to their differential stabilization. Finally, a faint band corresponding to a protein of the same size as Tsr was consistently seen in strain VB13, which lacks Tsr, Tar, Tap and Trg (compare the VB13, VB13/pMK113 and VB13/pAD56 lanes in Fig. 2). As the only other chemotactic signal transducer identified in *E. coli*, the oxygen receptor Aer, runs with an apparent molecular weight of ≈ 55 kDa (Bibikov *et al*., 1997), this phantom band

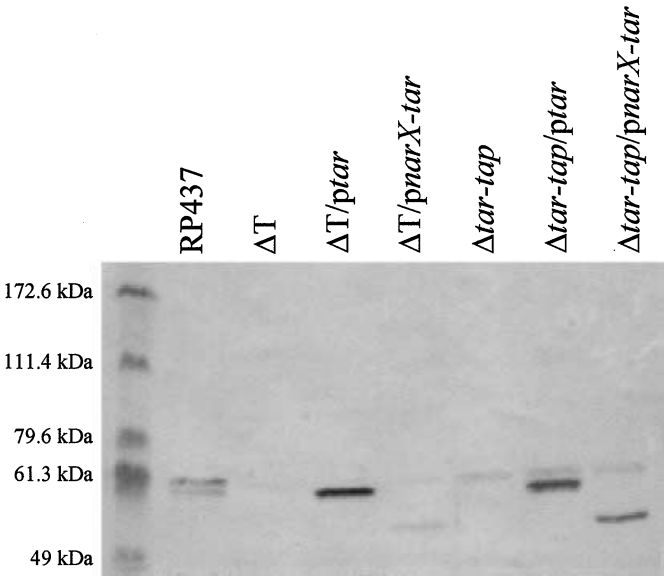

Fig. 2. Levels of NarX, Tar and Nart in strains VB13 (ΔT) and MM509 (Δ*tar–tap*). Immunoblots of proteins encoded by plasmid-borne *tar* (pMK113) or *narX–tar* (plasmid AD56) genes were determined in immunoblots developed with antibody against a highly conserved region from the cytoplasmic domain of the Tsr (serine) chemoreceptor. Proteins in membrane extracts from equal numbers of cells were loaded in each lane and separated by SDS–PAGE. An extract from the parental RP437 wild-type strain was included to indicate the normal levels of Tsr and Tar produced from the chromosomal genes. A faint band of unknown provenance appears in most lanes between the positions occupied by Tsr and Tar. The Nart hybrid migrates to a position corresponding to an apparent molecular mass of 55 kDa. Molecular weight markers are shown in the leftmost lane.

Table 1. Rotational biases of tethered cells.

Strain	Percentage time in CCW rotation[a]
VB13	99.5 ± 0.5
VB13/pAD56	91 ± 1
MM509	71 ± 5
MM509/pAD56	20 ± 2

a. The values shown are the means of the percentage time spent rotating CCW out of 60 s, ± the standard error. Data from 20 cells were averaged for each strain.

is unlikely to be Aer, and its provenance remains a mystery.

Behaviour of strain VB13/pAD56 on swarm plates

Plasmid pAD56 allowed strain VB13 to form a spreading colony in TB semi-solid agar, whereas plasmid pAD48 did not. VB13 colonies do not spread because the absence of chemoreceptors renders them exclusively smooth swimming. (The formation of a spreading colony in semi-solid agar requires cells that are able to run and tumble; Wolfe and Berg, 1989.) Thus, Nart must stimulate the activity of CheA kinase to produce enough CheY-P to induce tumbling, which corresponds to clockwise (CW) flagellar rotation.

The spreading colonies did not form chemotactic rings in semi-solid TB agar containing various concentrations of nitrate or nitrite. This result indicates that cells producing Nart as their sole transducer do not respond to any of the normal attractants for *E. coli* that are present in TB (e.g. amino acids). However, it does not indicate that these cells do not respond to nitrate or nitrite. The formation of chemotactic rings requires not only that a compound is an attractant but also that its metabolism creates a relatively steep gradient whose concentration increases away from the point of inoculation.

Behaviour of unstimulated tethered cells

The flagella of tethered cells of strain VB13/pAD48 were counterclockwise (CCW) biased (≈ 90% CCW flagellar rotation), although they did reverse, unlike the flagella on tethered cells of strain VB13, which rotated almost exclusively CCW (Table 1). The relatively low level of Nart present in these cells could explain the CCW bias. Tethered MM509 cells (60–80% CCW flagellar rotation) behaved essentially like wild-type cells (data not shown), whereas the flagella of MM509/pAD56 cells were substantially more CW biased (only 20% CCW flagellar rotation). These results support the notion that Nart facilitates random spreading of VB13 cells in semi-solid TB agar by increasing their tumbling frequency.

Responses of tethered cells to the addition of repellents

The adaptation times for tethered cells exposed to repellents are compiled in Table 2. RP437 (wild-type) cells gave brief (≈ 30 s) CW responses to the addition of 10^{-3} M leucine (sensed by Tsr) or Ni^{2+} (sensed by Tar). The adaptation times decreased at lower concentrations. As expected, MM509 cells (Δ*tar–tap*) did not respond to the addition of 10^{-3} M Ni^{2+}. However, they also did not respond to the addition of 10^{-3} M leucine. (Note that responses of <10 s would not be recorded.) The introduction of pMK113 into MM509 restored a remarkably long CW response

Table 2. Responses of tethered cells to the addition of repellents.

	Time of CW-only rotation (s) until the first CW → CCW reversal after repellent added at the indicated concentration (M)								
Leucine added	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Nickel added	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Strains					Strains				
MM500	28 ± 1	10 ± 0.8	6 ± 0.8	5 ± 0.4	MM500	26 ± 2	19 ± 1	5 ± 0.4	3 ± 0.3
MM509	0	0	0	0	MM509	ND	ND	ND	ND
MM509/pTar	5 ± 0.4	2 ± 0.3	0	0	MM509/pTar	480 ± 19	350 ± 25	25 ± 2	0
MM509/pAD56	0	0	0	0	MM509/pAD56	0	ND	ND	ND
VB13	0	0	0	0	VB13	0	0	0	0
VB13/pTar	0	ND	ND	ND	VB13/pTar	740 ± 28	610 ± 26	2 ± 0.4	0
VB13/pAD56	ND	ND	ND	ND	VB13/pAD56	ND	ND	ND	ND
Nitrate added	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Nitrite added	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Strains					Strains				
MM500	0	ND	ND	ND	MM500	0	ND	ND	ND
MM509	ND	ND	ND	ND	MM509	ND	ND	ND	ND
MM509/pTar	ND	ND	ND	ND	MM509/pTar	ND	ND	ND	ND
MM509/pAD56	0	ND	ND	ND	MM509/pAD56	0	ND	ND	ND
VB13	0	0	0	0	VB13	0	0	0	0
VB13/pTar	0	ND	ND	ND	VB13/pTar	0	ND	ND	ND
VB13/pAD56	84 ± 7	11 ± 1	6 ± 1	0	VB13/pAD56	10 ± 0.6	11 ± 1	6 ± 1	0

ND, not determined.

(480 s) to Ni^{2+}. This effect was even more pronounced with VB13/pMK113 cells, which had a CW response of 740 s after the addition of 10^{-3} M Ni^{2+}. However, MM509 cells containing pMK113 did not show a significant CW response to the addition of leucine. As expected, the pAD56 plasmid had no measurable effect on the responses to leucine or Ni^{2+} addition in either MM509 or VB13.

MM509 cells responded for a longer time (690 s) than RP437 cells to the addition of 10^{-3} M serine. Thus, a reduced level of Tsr, the absence of Tar, possible polarity of the *tar–tap* deletion on the expression of the downstream *cheRBYZ* genes, or some combination thereof, lowered the sensitivity, or accelerated the adaptation, to a step increase in the repellent leucine. These same factors may have heightened the sensitivity, or slowed the adaptation, to a step increase in the attractant serine.

Neither nitrate nor nitrite evoked a detectable response when added to tethered cells of strains RP437 (wild type), VB13 or MM509 that lacked pAD56. In contrast, when tethered cells containing pAD56 were exposed to nitrate or nitrite, both induced a period of exclusively CW flagellar rotation (Table 2). The threshold concentrations required for this response with VB13/pAD56 cells were 10^{-5} M for nitrate and 10^{-3} M for nitrite. The longest mean adaptation times (110 s for nitrate and 37 s for nitrite) were seen at 10^{-2} M. The responses became shorter at 10^{-1} M for both ions, perhaps because, at these high concentrations, the health of the cells was compromised. Tethered MM509/pAD56 cells also responded to the addition of nitrate and nitrate by turning their flagella only CW. The longest mean adaptation times (90 s for nitrate and 48 s for nitrite) were also observed at 10^{-2} M. The threshold concentration for the response to nitrite was again 10^{-3} M, but nitrate did not elicit a response at concentrations below 10^{-2} M. We currently have no good explanation for this somewhat surprising result.

The addition of leucine produced CW flagellar rotation in MM509 and MM509/pAD56 cells, but not in VB13 or VB13/pAD56 cells, as expected. The longest response was seen after the addition of 10^{-1} M leucine, the highest concentration tested. It was of the same duration (37 and 38 s respectively) in both strains. However, the threshold concentrations needed to generate a measurable response were very different: $<10^{-6}$ M with MM509 and 10^{-3} M with MM509/pAD56.

Responses of tethered cells to the removal of nitrate and nitrite

The physiologically relevant response of *E. coli* cells to repellents is to decrease their tumble frequency as the concentration of a repellent drops (Berg and Tedesco, 1975). Therefore, we looked at the behaviour of tethered cells upon removal of nitrate and nitrite. The response was dramatic. VB13/pAD56 cells responded with extended periods of CCW rotation with thresholds of 10^{-5} and 10^{-3} M for nitrate and nitrite respectively. The corresponding peak adaptation times, both at 10^{-2} M, were 1100 and 420 s. Adaptation times dropped off somewhat at 10^{-1} M but, in this case, an alternative explanation to cell damage at high anion concentration is that the 15 s flow did not remove all the nitrate or nitrite.

MM509/pAD56 cells behaved in a basically similar way (Table 2), but they exhibited a lower threshold (both elicited significant CCW responses at 10^{-6} M, the lowest concentration tested), and the peak adaptation times, although still at 10^{-2} M for both compounds, were slightly shorter than with VB13/pAD56 (790 s versus 1100 s for nitrate and 380 s versus 420 s for nitrite). In MM509 cells with or without pAD56, removal of leucine also evoked a CCW response, but it was much briefer (adaptation times after dilution from 10^{-2} M of 66 and 79 s in the absence and presence of pAD56). Although the plasmid did not significantly affect the maximum adaptation time to leucine, it did shift the threshold significantly, from $<10^{-6}$ M in MM509 cells without the plasmid to 10^{-3} M in MM509/pAD56. Here, one could argue that the strong CW bias of MM509/pAD56 cells (Table 1) reduced the adaptation time upon removal of leucine.

Repellent-in-pond capillary assays

The repellent-in pond assay (Tso and Adler, 1974) provides a direct method of demonstrating negative chemotaxis. This assay is a variant of the standard chemotaxis assay, in which cells enter a capillary as they move up a diffusion gradient of an attractant (Adler, 1973). The operating principle is that cells suspended in buffer containing a repellent will flee into capillary tubes filled with repellent-free buffer. With strain MM509, when 10^{-2} M leucine or acetate (both sensed by Tsr) was present in the pond, cells accumulated in the capillaries to four- or sixfold higher levels, respectively, than in the buffer controls (Fig. 3A). Neither nitrate nor nitrite in the pond led to increased accumulation of cells in the capillaries.

When strain MM509/pNarX-Tar was tested, cells accumulated to densities three- and fivefold higher than the buffer control when 10^{-4} M nitrate or 10^{-3} M nitrite (the concentrations that gave the peak responses) was present in the pond (Fig. 3B). Thus, nitrate and nitrate both act as repellents when Nart is expressed in strain MM509 and are effective at lower concentrations than leucine or acetate. As in the tethered cell assay, the presence of Nart desensitizes strain MM509 to leucine (and also to acetate). VB13/pAD56 cells responded to nitrite essentially the same as the MM509/pAD56 strain (Fig. 3C),

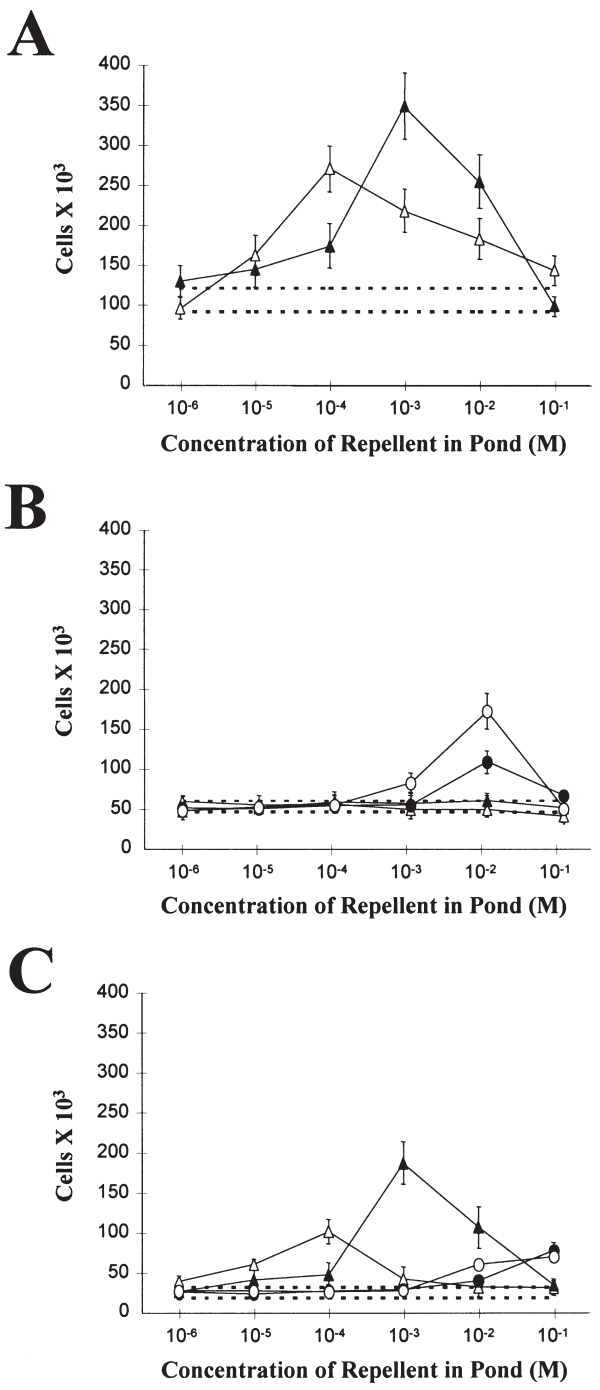

Fig. 3. Repellent-in pond capillary assays. The accumulation of MM509 cells harbouring (A) pMK113 (*tar*$^+$) or (B) pAD56 or of VB13 cells harbouring pAD56 (C) in capillaries containing buffer only is shown. The cells in the pond were resuspended in buffer containing the indicated concentrations of the repellents acetate (open circles), leucine (closed circles), nitrate (open triangles) and nitrite (closed triangles). The horizontal dashed lines show the mean background accumulation of cells, ± one standard deviation, when the pond also contained buffer only. Capillary assays were performed twice with triplicate samples according to the method of Adler (1973) as modified for repellent chemotaxis (Tso and Adler, 1974). The error bars on the data points indicate one standard deviation about the mean.

although their response to nitrate was weaker. As Tsr is absent in strain VB13, neither leucine nor acetate caused cells to accumulate in the capillaries.

Discussion

Nitrate and nitrite both stimulate autophosphorylation of NarX. This response is consistent with the induction of a transient period of CW flagellar rotation (tumbling) when these ions bind to the NarX(218)–Tar chimera (Nart), as CW rotation requires higher levels of *CheY-P* produced by increased CheA kinase activity. The responses to the addition and removal of nitrate and nitrate by MM509(Δ*tar–tap*)/pAD56 and VB13(ΔT)/pAD56 cells resemble those seen after the addition and removal of Ni^{2+} from MM509/pMK113(*tar*$^+$) and VB13/pMK113 cells. This similarity suggests that nitrate and nitrite elicit bona fide repellent responses. Furthermore, the ability of pAD56 to support CW flagellar rotation in strain VB13 demonstrates that the hybrid receptor must be able to interact with CheW and CheA in a productive manner. The avoidance of nitrate and nitrite in the repellent-in-pond capillary assay by MM509 and VB13 cells expressing Nart reinforces the conclusion that the NarX–Tar fusion protein mediates normal sensing, signalling and adaptation.

Nitrate typically elicited responses at lower concentrations than nitrite, and the peak responses induced by nitrate were longer than those seen with nitrite. Thus, nitrate appears to have a higher affinity for Nart than nitrite does, and it evokes a more robust response. This same relative efficacy of nitrate and nitrite has been observed with NarX itself (Williams and Stewart, 1997; Lee *et al.*, 1999; Wang *et al.*, 1999), bolstering our confidence that NarX and Nart bind ligands and carry out transmembrane signalling in a similar fashion.

The NarX(218)–Tar hybrid joins residues 1–218 of NarX to residues 257–553 of Tar. The fusion joint is at the end of the linker region, which contains the HAMP domain. The HAMP domain is highly conserved in a large family of homodimeric transmembrane receptors that couple ligand binding to conformational changes which alter the signal produced by the receptor (Aravind and Ponting, 1999). The NarX(270)–Tar hybrid fuses residues 1–270 of NarX, which still precede the conserved sensor kinase signalling region of NarX, to residues 257–553 of Tar. The translation product was not detected on immunoblots, presumably because it is unstable. This second hybrid was constructed to test whether a second region of extended amphipathic helical structure, similar to the one preceding the fusion joint in Nart, could also be coupled to Tar to generate a functional chemoreceptor. The instability of the protein limits the inferences that can be drawn, but fusions clearly cannot be made at any arbitrary point and still generate an active chimeric protein.

The Taz (Utsumi *et al*., 1989) and Trz (Baumgartner *et al*., 1994) proteins couple the ligand-binding domain of Tar or Trg to the kinase domain of EnvZ. The fusion joints in these proteins are in the same relative position with respect to the linker regions and HAMP domains as in Nart. Both proteins mediate enhanced transcription of *ompC*, which requires increased levels of phospho-transfer to OmpR from the signalling domain of EnvZ, in response to their respective ligands.

Taz clearly changes its signalling behaviour in response to aspartate, and Trz alters its signalling behaviour in response to ribose and galactose. However, several limitations in the results reported for these two proteins prevent those authors from making an airtight case for a common mechanism of transmembrane signalling by chemoreceptors and sensor kinases. (i) As attractants inhibit the activity of CheA kinase, one might expect that attractants should decrease levels of Taz or Trz autophosphorylation, and therefore decrease levels of OmpR-P. The opposite effect is observed. (Aspartate is proposed to increase OmpR-P levels by inhibiting a phosphatase activity of EnvZ, but a similar effect on CheA has not been observed with chemoreceptors.) (ii) Although the ligand-occupied ribose and galactose/glucose-binding proteins elicit an increase in OmpC expression, ligand-occupied maltose-binding protein does not have this effect. The reason for this apparent discrepancy has not been explained. (iii) The role of Taz and Trz on the expression of the *ompF* gene, which should go down when OmpR-P concentrations rise to a level that induces *ompC* expression, has not been reported. (iv) Finally, EnvZ has no known ligand, so the function of reciprocal constructs fusing the EnvZ sensing domain to a chemoreceptor cytoplasmic signalling domain cannot be addressed.

We are in the process of testing a reciprocal Tar–NarX hybrid (Tarx), which fuses residues 1–256 of Tar to residues 219–598 of NarX, for its ability to phosphorylate the NarL (or NarQ) response regulator and to modify this activity in response to the Tar attractant ligands aspartate and maltose and the Tar repellent ligands Ni^{2+} and Co^{2+}. We predict that cells expressing Tarx will respond to aspartate, and perhaps maltose, by decreasing the expression of genes whose transcription requires NarL-P and increasing the expression of genes whose transcription is repressed by NarL-P. Conversely, Ni^{2+} and Co^{2+} should induce the transcription of the first set of genes and repress the expression of the second. It has already been proposed, based on mutational analysis, that NarX and Tsr share a transmembrane signalling function (Collins *et al*., 1992). Demonstration that repellents and attractants sensed by Tar regulate the activity of Tarx in the expected manner will solidify our conclusion that transmembrane signalling operates by fundamentally the same mechanism for bacterial chemoreceptors and sensor kinases.

VB13/pAD56 cells, which overexpress Nart, gave a longer than normal repellent (CW) response (84 s) to the addition of 10^{-3} M nitrate. We presume that CheB methylesterase becomes limiting under these conditions, as adaptation to repellents involves demethylation of the receptors. This effect was considerably exaggerated when 10^{-3} M Ni^{2+} was added to VB13/pMK113 cells that overexpress Tar, in which the adaptation time was 740 s. This difference could result from the higher level of expression of Tar compared with Nart (Fig. 2). When MM509 cells containing the same two plasmids were tested, however, there was no detectable CW response to adding 10^{-3} M nitrate, and the CW response to 10^{-3} M Ni^{2+} decreased to 480 s. Thus, the presence of Tsr apparently accelerates adaptation to non-cognate repellents under these conditions. When repellents were removed (Table 3), very long CCW responses were seen with both nitrate and Ni^{2+}, but here the differences between the VB13 and MM509 strains were much less pronounced. In this case, the extended adaptation times could result from a shortage of CheR methyltransferase, which sets the rate of adaptive methylation after the addition of attractant.

The effects of the expression level of Tar and Nart on responses mediated by the chromosomally encoded Tsr receptor, although not the focus of this work, beg comment. The absence of Tar in Δ*tar–tap* strain MM509 lengthened the period of exclusively CCW rotation (the adaptation time) after the addition of a saturating (10^{-3} M) concentration of serine (Table 3), and overproduction of Tar in MM509/pMK113(*tar*$^+$) decreased the adaptation time. However, the absence of Tar decreased the adaptation time after the addition of 10^{-5} M serine, and overproduction of Tar results in relatively longer adaptation times at low serine concentrations. In contrast, overproduction of Nart in strain MM509/pAD56(*NarX218–Tar*) decreased the adaptation time after the addition of serine (420 and 22 s at 10^{-3} and 10^{-4} M, respectively, compared with 470 and 220 s for RP437 wild-type cells).

A totally different pattern was seen with the repellent leucine, which induces CW rotation upon addition and CCW rotation upon removal. The longest adaptation times to leucine addition (28 s at 10^{-3} M) were seen with strain RP437, and strain MM509, with or without the pMK113 or pAD56 plasmids, exhibited essentially no response to leucine addition (Table 2). The longest adaptation times after the removal of high concentrations of 10^{-3} M leucine were seen with MM509/pMK113 cells, and the adaptation times became progressively shorter in strains RP437 and MM509 (Table 3). Strain MM509/pAD56 did not respond at all. However, the relative reduction in adaptation times as the initial leucine concentration decreased was much

Table 3. Responses of tethered cells to the addition of attractants or removal of repellents.

	Time of CCW-only rotation (s) until the first CCW → CW reversal after addition of attractant at the indicated concentration (M) or replacement of repellent present at the indicated concentration with unadulterated buffer								
Serine added	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Leucine removed	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Strains					Strains				
MM500	470 ± 18	230 ± 3	100 ± 2	0	MM500	64 ± 2	62 ± 2	50 ± 1	43 ± 1
MM509	690 ± 8	340 ± 4	60 ± 2	1 ± 0.3	MM509	31 ± 1	13 ± 1	10 ± 1	9 ± 0.6
MM509/pTar	180 ± 5	41 ± 3	30 ± 2	14 ± 2	MM509/pTar	120 ± 3	50 ± 3	24 ± 2	6 ± 1
MM509/pAD56	420 ± 8	22 ± 2	ND	ND	MM509/pAD56	3 ± 0.5	ND	ND	ND
VB13/pTar	0	ND	ND	ND	VB13/pTar	0	ND	ND	ND
VB13/pAD56	0	ND	ND	ND	VB13/pAD56	0	ND	ND	ND
Aspartate added	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Nickel removed	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Strains					Strains				
MM500	340 ± 6	230 ± 6	100 ± 4	6 ± 0.8	MM500	140 ± 3	130 ± 2	53 ± 1	44 ± 1
MM509	0	ND	ND	ND	MM509	0	ND	ND	ND
MM509/pTar	1130 ± 32	1030 ± 24	690 ± 20	540 ± 10	MM509/pTar	650 ± 18	600 ± 18	120 ± 9	39 ± 3
MM509/pAD56	0	ND	ND	ND	MM509/pAD56	0	ND	ND	ND
VB13/pTar	930 ± 21	710 ± 10	400 ± 12	105 ± 7	VB13/pTar	680 ± 11	600 ± 8	65 ± 6	38 ± 2
VB13/pAD56	0	ND	ND	ND	VB13/pAD56	0	ND	ND	ND
Nitrate removed	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Nitrite removed	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Strains					Strains				
MM500	0	ND	ND	ND	MM500	0	ND	ND	ND
MM509	0	ND	ND	ND	MM509	0	ND	ND	ND
MM509/pTar	0	ND	ND	ND	MM509/pTar	0	ND	ND	ND
MM509/pAD56	740 ± 13	300 ± 6	110 ± 4	52 ± 4	MM509/pAD56	0	ND	ND	ND
VB13/pTar	0	ND	ND	ND	VB13/pTar	0	ND	ND	ND
VB13/pAD56	990 ± 15	880 ± 25	120 ± 6	ND	VB13/pAD56	110 ± 4	ND	ND	ND

ND, not determined.

more abrupt in strain MM509/pMK113 than in either strain RP437 or strain MM509.

These results are complicated enough to defy a simple explanation, at least by us. It seems likely that some combination of effects contributes to these phenomena: (i) titration of limiting Che proteins (the CheR methyltransferase and CheB methylesterase, whose competing activities regulate chemotactic adaptation, are good candidates); (ii) the effect of the level of receptor methylation in the receptor–CheW–CheA tertiary complex on ligand affinity; and (iii) higher order associations of different receptors and Che proteins within the receptor patch (Maddock and Shapiro, 1993; Shimizu *et al*., 2000; Sourjik and Berg, 2000; 2002). These data reinforce the conclusion of Barak and Eisenbach (2001) that the response to attractant addition is mechanistically different from the response to repellent removal. However, much more thought and more experimentation will be required to untangle this particular Gordian knot.

Returning to the main theme after this digression, the ability of the Nart hybrid to function like a canonical homodimeric chemoreceptor may herald an opportunity to design novel receptors and sensor kinases by a mix-and-match approach. The chemical recognition domains (including those of the cognate periplasmic-binding proteins) of such designer receptors can potentially be modified to recognize novel ligands (Hellinga and Marvin, 1998). These engineered signal transduction systems can, in principle, be coupled to a wide variety of outputs and may prove to be of considerable utility.

Experimental procedures

Media

Routine media were prepared according to the method of Miller (1972). Tryptone broth (TB) is 1% (w/v) tryptone extract and 0.8% (w/v) NaCl. Luria broth (LB) contains 1% tryptone extract, 0.5% (w/v) yeast extract, 0.5% NaCl. LB solid agar contains 1.5% (w/v) Difco agar, and TB swarm plates contain 0.325% (w/v) Difco agar. Liquid cultures and agar plates were incubated at 37°C for LB or 32°C for TB. Media were supplemented with ampicillin (Amp, 50 μg ml^{-1}) and tetracycline (Tet, 5 μg ml^{-1}) as needed.

Strains and plasmids

Escherichia coli strain RP437 is wild type for motility (Parkinson and Houts, 1982). Strain MM509 is an *eda*$^+$ Δ*tar-tap*5201 derivative of strain RP437 (Gardina *et al*., 1992). Strain VB13 is a *thr*$^+$ *eda*$^+$ *tsr*7021 *trg*::Tn*10* Δ*tar-tap*5201 derivative of RP437. Plasmid pMK113 contains the *E. coli tar* gene and the single-stranded origin of phage M13 from plasmid pZ150 (Gardina *et al*., 1992).

We constructed plasmids encoding two hybrid proteins, NarX(218)–Tar and NarX(270)–Tar, each of which contains a different number of N-terminal residues of NarX fused to the C-terminal cytoplasmic adaptation and signalling regions of Tar. NarX(218)–Tar, referred to as Nart, contains residues 1–218 of NarX fused to residues 257–553 of Tar. NarX(270)–Tar contains residues 1–270 of NarX fused to residues 257–553 of Tar. The *narX* sequences for both fusions were obtained by polymerase chain reaction (PCR) using pLK633 (Kalman and Gunsalus, 1990) as a template.

To construct the *NarX(218)–Tar* gene, codon 218 (Asn) of *narX* was converted to a CAT (His) codon by site-directed mutagenesis. This mutation created an *Nde*I site (CATATG) 44 codons after the DNA sequence encoding the second transmembrane helix of NarX. A 0.7 kbp PCR fragment of pLK633 was obtained that contained codons 1–218 of *narX* flanked on the 3′ end by the introduced *Nde*I site and on the 5′ end by a region corresponding to the sequence upstream of the *tar* start codon up to a *Bam*HI site in pMK113 (Gardina *et al*., 1992), which is downstream of the native *tar* promoter. This PCR product was cut with *Bam*HI and *Nde*I and ligated into a 5.3 kbp *Bam*HI–*Nde*I fragment from pMK113 generated by digestion with *Bam*HI and partial digestion with *Nde*I. (There is a second *Nde*I site in pMK113 downstream of *tar*.) This ligation yielded plasmid pAD56, which should transcribe *NarX(218)–Tar* from the modified *tar* promoter of pMK113.

NarX(270)–Tar was constructed using a similar strategy. The only difference was that the *Nde*I site was created at codons 270 (Pro) and 271 (Val), which were converted to the CAT (His) and ATG (Met) codons by site-directed mutagenesis. A 0.8 kbp fragment from pLK633 was obtained that contained codons 1–270 of *narX*, again flanked on the 3′ end by the introduced *Nde*I site and on the 5′ end by the same sequence upstream of the *tar* start codon up to the *Bam*HI site. This PCR product was ligated into the 5.3 kbp *Bam*HI–*Nde*I fragment of pMK113 to yield plasmid pAD48, which should also transcribe *NarX(270)–Tar* from the modified *tar* promoter.

Immunoblotting

Cultures were grown in TB with swirling in 125 ml Erlenmeyer flasks. Overnight cultures were diluted 1:100 (v/v) into a 10 ml volume of TB. Cells were harvested at an A_{590} of 0.8. An equal number of cells from the exponential phase culture was pelleted and washed once with TE buffer (10 nM Tris, pH 7.5, 0.1 mm EDTA), and the cells were resuspended in 50 µl of loading buffer [2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 8.5% (v/v) glycerol, 60 mm Tris, pH 6.8, 0.0004% (w/v) bromophenol blue]. Freeze–thaw extracts were prepared from these resuspended cells by three alternating cycles of 5 min of boiling and 5 min on ice. Proteins were separated by 12% acrylamide SDS–PAGE and transferred to nitrocellulose. Antibody against the conserved cytoplasmic domain of Tsr (Ames and Parkinson, 1994) was used to probe the immunoblots, and cross-reacting proteins were visualized with alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad). The blot was developed using SigmaFast (Sigma).

Swarm plate assays

The motility of each strain was assessed by inoculating colonies onto TB swarm plates. Plates were incubated at 32°C for 6–8 h, and the swarm diameters were measured and recorded.

Tethered cell assays

The flagellar filaments of highly motile cells were sheared to short stubs, and the cells were mixed with antifilament antibody and tethered to glass coverslips (Silverman and Simon, 1974). These coverslips were affixed to a flow chamber (Berg and Block, 1984), which was used to introduce chemoeffectors to the tethered cells. Sensitive cells responded to repellents with a brief period of exclusively clockwise (CW) flagellar rotation before adapting and returning to baseline reversing behaviour. Removal of repellent after the introduction of buffer induced a much longer period of exclusively counterclockwise (CCW) flagellar rotation in sensitive cells, again followed by a return to reversing behaviour. The adaptation time was measured from the instant that flow was initiated, leading to a systematic overestimation (by a few seconds) of the adaptation time, as several seconds are required for the new medium to reach the cells. Cells that responded for <15 s were scored as non-responders (0 s) because the direction of rotation cannot be scored reliably during the flow. The responses of at least 20 cells were averaged for each measurement. All experiments with tethered cells were carried out at room temperature (22–24°C).

Repellent-in-pond capillary assay

Cells were placed in a pond containing repellents at various concentrations. A capillary filled with buffer was inserted into each pond. After 1 h of incubation at 30°C, the capillary contents were blown out, serially diluted and plated on LB agar. The number of cells within the capillary was calculated from colony counts. Capillary assays were carried out in triplicate according to the method of Adler (1973) as modified for repellent chemotaxis (Tso and Adler, 1974).

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