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Inter-receptor communication through arrays of bacterial chemoreceptors

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The sensing mechanisms of chemotactic bacteria allow them to respond sensitively to stimuli. *Escherichia coli*, for example, respond to changes in chemoattractant concentration of less than 10% over a range spanning six orders of magnitude[1,2]. Sensitivity over this range depends on a nonlinear relationship between ligand concentration and output response[3]. At low ligand concentrations, substantial amplification of the chemotactic signal is required; however, the mechanism responsible for this amplification remains unclear. Here we demonstrate that inter-receptor communication within a lattice[4,5] acts to amplify and integrate sensory information. Synthetic multivalent ligands that interact through the low-abundance, galactose-sensing receptor Trg stabilize large clusters of chemoreceptors and markedly enhance signal output from these enforced clusters. On treatment with multivalent ligands, the response to the attractant serine is amplified by at least 100-fold. This amplification requires a full complement of chemoreceptors; deletion of the aspartate (Tar) or dipeptide (Tap) receptors diminishes the amplification of the serine response. These results demonstrate that the entire array is involved in sensing. This mode of information exchange has general implications for the processing of signals by cellular receptors.

A family of at least four chemoreceptors (methyl-accepting chemotaxis proteins, MCPs) mediates metabolism-independent chemotactic responses in *E. coli*[6,7]. The high-abundance MCPs (Tsr and Tar) mediate responses to serine and aspartate, respectively; the low-abundance MCPs (Tap and Trg) are present at only 10% of the concentration of Tsr[8,9]. Responses to dipeptides are transmitted through Tap. Trg mediates responses to galactose and ribose by means of their respective periplasmic binding proteins. The MCPs transmit information through a two-component signalling cascade. These transmembrane proteins exist as dimers that complex to the cytoplasmic kinase CheA and the adapter protein CheW[10,11]. Ligand binding to the MCPs, either alone or together with one of the periplasmic binding proteins, promotes a conformational change in the receptor that influences the activity of CheA[12,13]. CheA transfers a phosphoryl group to CheY, the response regulator that ultimately controls flagellar rotation and locomotion. When the attractant concentration remains stable, *E. coli* adapt through a process that involves methylation of glutamate residues in the cytoplasmic domains of the MCPs. The MCPs and other components of the signalling cascade localize to the cell poles of *E. coli*[14]. This pattern of MCP localization is conserved in other bacteria and in *Archaea*[15].

Recent models implicate inter-receptor communication within chemoreceptor arrays in signal amplification[4,5]. Such a mechanism would complement findings indicating that inter-receptor transfer of methyl groups occurs during adaptation to chemotactic stimuli[16]. These results and others[17] support inter- and intradimer cross-talk during adaptation. With regard to signal amplification, however, the evidence supporting a role for chemoreceptor arrays is indirect. One proposal is that inter-receptor communication occurs between homodimers of one receptor-type[18,19]. This homodimer–homodimer communication may be mediated by transmission of ligand-induced conformational changes facilitated by receptor–receptor contact[13,20]. However, it is unclear what mechanisms of inter-receptor communication function during excitatory chemotactic responses in the entire MCP array. In lattice models of chemoreceptor function, the proximity of MCPs is a principal determinant of signalling[4,5]. We proposed, therefore, that the stabilization of receptor–receptor contacts would reveal the importance of this parameter for the amplification of chemotactic signals.

To create reagents that stabilize inter-receptor interactions, we synthesized multivalent derivatives of the chemoattractant galactose by ring-opening metathesis polymerization (ROMP) (Fig. 1a). ROMP allows the generation of multivalent ligands with distinct valencies[21]. Ligands of sufficient length (approximately 25 monomer units) would be expected to possess the ability to bind multiple MCPs simultaneously[22]. Galactose and synthetic galactose-bearing ligands[23] activate Trg through interaction with the periplasmic binding protein, glucose/galactose-binding protein (GGBP). We anticipated that the multivalent chemoattractants would stabilize inter-receptor contacts by forming complexes containing multiple copies of Trg, and thus could be used to elucidate the role of receptor position in signal amplification.

To investigate the impact of ligand valency on bacterial behaviour, we conducted motion analysis. The mean angular velocity of a bacterial population decreases in response to chemoattractants[24]. As expected[22], angular velocity decreased when galactose or galactose derivatives 1–3 were introduced to cells of the chemotactically active *E. coli* strain at 100 μM galactose (all concentrations were based on the total amount of galactose) (Fig. 1b). The magnitude of the response depended on the concentration and valency of the attractant (Fig. 1c). The adaptation time—the period before the population returns to pre-stimulus angular velocities—was also influenced by these factors (see Supplementary Information). Notably, multivalent ligand 3 was the most potent chemoattractant. This attractant was 100- to 1,000-fold more potent than its monovalent counterpart ligand 1. In contrast, multivalent ligand 2 did not have a significantly different chemotactic activity compared to monovalent ligand 1 or galactose at most concentrations. Molecular modelling studies indicate that multivalent ligand 2 is of insufficient length to cluster Trg[23]. These data suggest that the differences in chemotactic activity between ligands 3 and 1 are due to the ability of ligand 3 to stabilize MCP clusters.

To test whether the synthetic chemoattractants alter MCP clustering, we used fluorescence microscopy to monitor receptor position[22]. MCPs distribute randomly in *E. coli* RP1078 (ref. 25), a strain that lacks functional CheW[14,22]. Multivalent ligand 3, but not monovalent ligand 1, induces chemoreceptor clustering in this *cheW* mutant (Fig. 1d). The patterns of MCP distribution in cells

treated with multivalent ligand 2 were indistinguishable from those treated with monovalent ligand 1 (data not shown). Thus, only ligand 3, which is predicted to interact through multiple copies of Trg, promotes clustering of the chemoreceptors[22].

The chemoreceptor clusters that formed in the presence of multivalent ligand 3 were larger than would be expected for those only composed of Trg (approximately 150 copies per cell)[8]. We propose that these clusters are composed of heterogeneous collections of MCPs. The cytoplasmic and transmembrane domains that mediate inter-MCP contact are homologous (>50% sequence identity). Heterologous MCP clustering may therefore be mediated through Trg–MCP interactions, which are encouraged by multivalent ligand 3. Because Tsr is the MCP with the highest cellular concentration (roughly 3,000 copies per cell)[9], it should constitute a large percentage of the receptors in clusters stabilized by multivalent ligand 3. Accordingly, we created a fluorescent ligand for the chemoreceptor Tsr (serine-fluorescein (Ser-fl); Fig. 2a) to probe Tsr location. When the *E. coli cheW* mutant was pre-treated with galactose derivatives 1 or 2 before Ser-fl addition, no change in MCP clustering was observed (see Supplementary Information). In contrast, when multivalent ligand 3 was added, the fluorescence due to Ser-fl was localized to clusters (Fig. 2b, top panel). These clusters co-localized with the MCPs (Fig. 2b, bottom panel). These data suggest that ligands for Trg (multivalent ligand 3) and Tsr (Ser-fl) bind to clusters of MCPs, indicating that both Trg and Tsr are included in the clusters reinforced by multivalent ligand 3.

Fluorescence and electron microscopy experiments indicate the MCPs are localized[14,15]; however, these methods cannot discern whether the distances between receptors are appropriate for inter-receptor communication. We tested whether Ser-fl could serve as a sensitive probe of changes in Tsr clustering by comparing the fluorescence emission intensity of cells treated with Ser-fl in the presence of monovalent ligand 1 or multivalent ligand 3. The fluorescence intensity due to Ser-fl binding to either *cheW* mutants (Fig. 2c, top panel) or wild-type cells (Fig. 2c, bottom panel) was unaffected by pre-treatment with monovalent ligand 1. In contrast, on pre-treatment with multivalent ligand 3, the fluorescence intensity decreased 25–30% in the *cheW* mutant (Fig. 2c, top panel) and 15–20% in wild-type cells (Fig. 2c, bottom panel). These results are consistent with fluorescence self-quenching resulting from the clustering of Ser-fl binding sites. Our observations suggest that multivalent ligand 3 serves to stabilize inter-receptor contacts in wild-type cells, albeit to a lesser extent than in *cheW* mutants.

If inter-receptor communication is critical for signal amplification, responses mediated by Tsr should be enhanced in the presence of a ligand that increases MCP proximity. To test this hypothesis, wild-type *E. coli* were treated with ligands 1–3 and then allowed to adapt. After full adaptation, serine was added, and the angular velocity was measured. Pre-treatment with multivalent ligand 3, but not monovalent ligand 1 or multivalent ligand 2, amplified the response of the bacteria to serine (Fig. 3a). The serine concentration required to achieve a behavioural response was decreased at least 100-fold by pre-treatment with multivalent ligand 3. Similarly, responses to aspartate were augmented by pre-treatment with

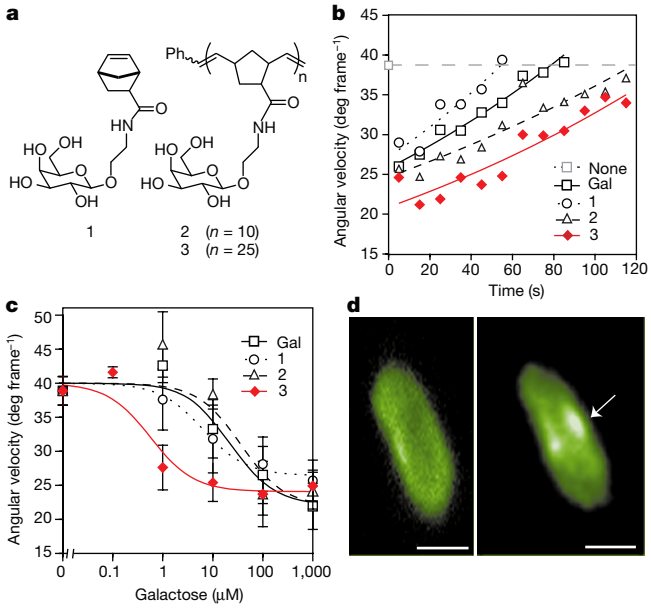

Figure 1 Effects of chemoattractants on the behaviour of *E. coli* and the localization of MCPs. **a**, Chemical structures of synthetic galactose-bearing monomer 1 and multivalent chemoattractants 2 and 3 (ref. 22). Ligand valency (*n*) represents the degree of polymerization, which was determined by integration of the ^{1}H nuclear magnetic resonance (NMR) spectrum. **b**, Plot of average angular velocity versus time for *E. coli* AW405 treated with chemoattractant (galactose or ligands 1–3). Motion analysis was performed as described previously (see Supplementary Information)[22,24]. The ligand concentration (100 μM) is given as the total concentration of galactose or galactose residues. Results are the average of at least four experiments performed in triplicate. **c**, Angular velocity of *E. coli* treated with various concentrations of chemoattractant. Data points represent the average angular velocity for the first 15 s after addition of attractant or buffer. **d**, Fluorescence micrographs of *cheW E. coli* RP1078 cells treated with galactose-bearing ligand 1 (left) or 3 (right). Microscopy was performed using fluorescein-labelled anti-MCP antibodies, as described[22]. A sample cluster is highlighted with an arrow. The average size of the clusters observed (10,000 ± 3,500 nm^2) suggests that they contain MCPs other than Trg. The theoretical maximum size of a cluster containing 150 copies of Trg[8,9] is approximately 2,900 nm^2 (ref. 20). Scale bar, 0.5 μm.

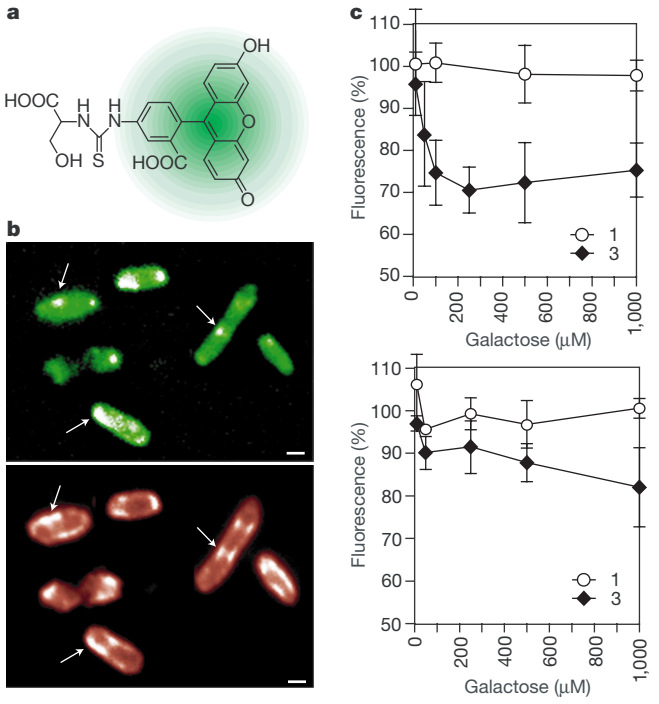

Figure 2 Tsr is included in clusters formed on treatment with galactose-bearing ligand 3. **a**, Chemical structure of the serine-fluorescein conjugate (Ser-fl). Ser-fl served as a chemoattractant in wild-type AW405 but not Tsr mutant AW518 cells (see Supplementary Information). **b**, Treatment with 500 μM galactose-bearing ligand 3 causes patching of Ser-fl binding sites (top). Patches (arrows) occur in both polar and non-polar locations and correspond to sites of anti-MCP antibody binding (bottom). Microscopy was performed as described previously[22]. Scale bar, 0.5 μm. **c**, Fluorescence intensity of *cheW* mutant RP1078 (top panel) or wild-type AW405 (bottom panel) cells after treatment with ligands 1 or 3, followed by 30 μM Ser-fl. The concentration of ligand 1 or 3 is reported as the concentration of galactose residues. Values (±s.e.) are the average of three independent experiments.

multivalent ligand 3 (Fig. 3c). To test whether this enhancement arises from attractant binding to clustered receptors, ribose—which also signals through Trg[2]—was added as a second attractant. If ribose displaces multivalent ligand 3, no signal enhancement should be observed. Even if ligand 3 remains bound, the low copy number of Trg should ensure that the clusters contain little if any unoccupied Trg. Accordingly, when ribose was the second attractant (Fig. 3c), no potentiation of response was observed. These results strongly support a role for inter-receptor communication in chemotactic signal amplification.

Next, we explored the contribution of various chemotactic signalling components to inter-receptor communication by analysing the responses of strains lacking one or more principal constituent. Multivalent ligand 3 had no effect on the responses to serine in a *ggbp* (*E. coli* AW550) or *trg* (*E. coli* AW701) mutant (see Supplementary Information). A *tsr* mutant (*E. coli* AW518)[26] also failed to respond to the combination of ligand 3 and serine, indicating that the serine receptor was required for amplification of the chemotactic response (see Supplementary Information). Deletion of Tar (*E. coli* RP2361) decreased the potentiation of serine responses from 36% to 15% (Fig. 4a). When both Tar and Tap (*E. coli* RP5854) are deleted, the potentiation decreases by an additional 5%. Thus, a full complement of MCPs is required for maximal potentiation of serine responses—even receptors that do not bind chemoattractant contribute to the response.

We proposed that multivalent ligand 3, by stabilizing inter-receptor contact, would amplify any tendency of MCPs to perform as a lattice during sensory stimulation. Given the enhancement of the serine response observed in the presence of ligand 3 and its dependence on the full complement of chemoreceptors, we sought to determine whether inter-receptor communication can be detected without the aid of the synthetic ligand. We tested whether *E. coli* mutants lacking an MCP class exhibit diminished chemotactic responses. MCP mutants were treated with the attractant serine, and their behavioural responses were assessed by motion analysis. Responses to 100 μM serine were decreased 12–14% in mutants lacking Tar or both Tar and Tap, relative to those in wild-type cells (Fig. 4b). Cells lacking Trg exhibited no detectable

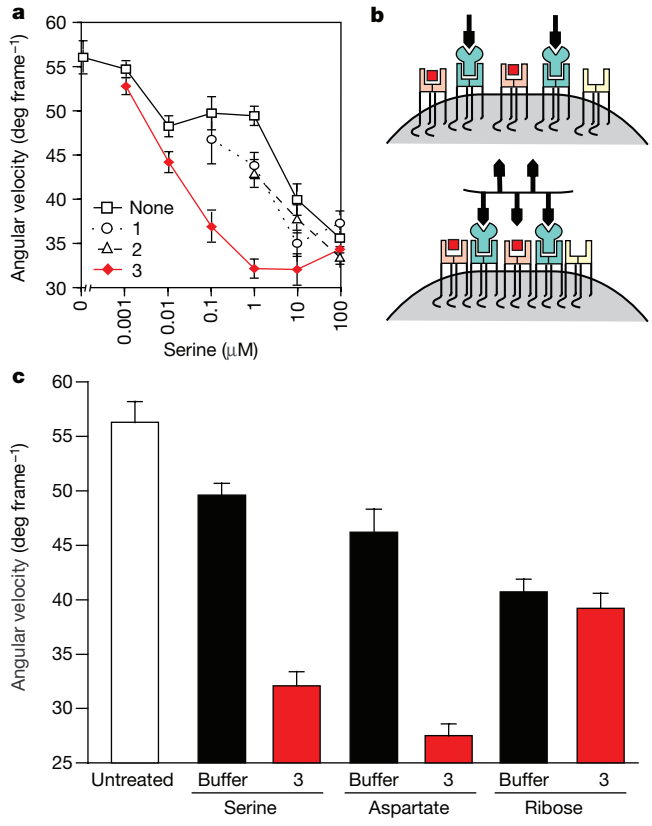

Figure 3 Response of wild-type *E. coli* (AW405) to chemoattractants after pre-treatment with buffer or ligands 1–3 at a galactose residue concentration of 10 μM. **a**, *Escherichia coli* were allowed to adapt fully to ligands 1–3 (120 s) at 30 °C after pre-treatment. Results represent the average angular velocity over the first 2 min after the addition of serine. Full adaptation to serine concentrations of 100 nM or greater required more than 2 min, a result consistent with the adaptation time of *E. coli* strain AW405 in response to 300 nM aspartate, determined by motion analysis[24]. Values (±s.e.) are the average of at least four experiments performed in triplicate. **b**, Schematic model for the stabilization of MCP clusters and potentiation of serine responses. After treatment with multivalent ligand 3 (bottom), clusters of MCPs are stabilized relative to MCPs treated with monovalent ligand 1 (top). Trg (green) and GGBP (green) are shown. Additional MCPs are represented by the pink (Tsr) and yellow (Tar) receptors. Serine (red) is shown bound to Tsr. **c**, Motion analysis results after pre-treatment with ligand 3 at a final galactose residue concentration of 10 μM. Values represent the average angular velocity over the first 2 min after addition of the second attractant (1 μM serine, 1 μM aspartate, or 10 μM ribose). Adaptation times were at least 2 min in each case. Values (±s.e.) are the average of at least four experiments performed in triplicate.

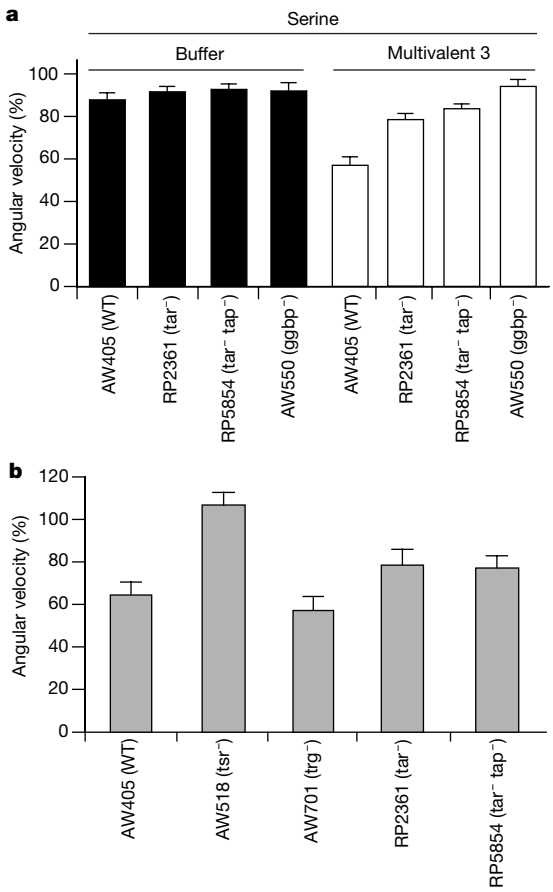

Figure 4 Response of *E. coli* to serine. **a**, Response of *E. coli* mutants to serine after pre-treatment with multivalent ligand 3 or buffer. Cells of *E. coli* strain AW405 (wild type, WT), RP2361 (tar⁻), RP5854 (tar⁻ tap⁻), or AW550 (ggbp⁻) were pre-treated with either ligand 3 (10 μM galactose residue concentration) or buffer. Serine was added to a final concentration of 1 μM after adaptation to the galactose-substituted ligand (120 s). Results are the average of the angular velocity collected during the 2 min after addition of serine. The data were normalized to a control in which buffer was used for both treatments. Values (±s.e.) are the average of at least four experiments performed in triplicate. **b**, Response of *E. coli* MCP mutants to serine without pre-treatment. Serine (100 μM) was added to cells of strain AW405 (WT), AW518 (tsr⁻), AW701 (trg⁻), RP2361 (tar⁻), or RP5854 (tar⁻ tap⁻). Motion analysis was performed for 2 min. Values (±s.e.) are the average of three experiments performed in triplicate.

decrease in serine responses, a result consistent with the expectation that low-abundance receptors make smaller contributions to signal amplification. Notably, high attractant concentrations were required to distinguish between wild-type and mutant responses (see Fig. 4a). This finding highlights the benefits of synthetic multivalent ligand 3, which is a highly sensitive probe of the role of chemoreceptor clustering. Together, our results suggest that inter-receptor interactions can mediate the signal amplification that is required for the dynamic range observed for chemotactic responses[1,3].

Despite the difference in receptor copy number, chemotactic responses towards galactose (through Trg) and serine (through Tsr) occur over comparable dynamic ranges[1,2]. Our finding that high- and low-abundance receptors can communicate to amplify signals indicates that information transfer occurs between these receptor classes. These results provide a mechanism for achieving parity among chemotactic responses despite the differences in receptor copy number. Our data complement the finding that intra-dimer methylation governs the dependence of the low-abundance receptors on the high-abundance MCPs for proper adaptation[16]. Thus, inter-receptor communication may be responsible for both signal amplification during excitatory responses and receptor methylation during adaptation.

Receptors are often localized to particular regions of the cell surface. Yet the significance of such localization is often obscure. We have shown that multivalent ligands can reveal the role of chemoreceptor arrays in governing cellular responses. Models for amplification through receptor arrays have been proposed for other signalling processes[27,28], including T-cell-receptor-mediated lymphocyte activation[29,30]. Our results indicate that synthetic multivalent ligands can serve as valuable probes of the mechanisms underlying signal amplification and integration. □

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Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction

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Bacteria of the genus *Enterococcus* are the main causes of highly antibiotic-resistant infections that are acquired in hospitals[1,2]. Many clinical isolates of *Enterococcus faecalis* produce an exotoxin called cytolysin that contributes to bacterial virulence[3]. In addition to its toxin activity, the cytolysin is bactericidal for nearly all Gram-positive organisms[4]. An understanding of conditions that regulate cytolysin expression has advanced little since its initial description[5]. Here we show that the products of two genes, *cylR1* and *cylR2*, which lack homologues of known function, work together to repress transcription of cytolysin genes. Derepression occurs at a specific cell density when one of the cytolysin subunits reaches an extracellular threshold concentration. These observations form the basis of a model for the autoinduction of the cytolysin by a quorum-sensing mechanism involving a two-component regulatory system.

The large and small subunits of the cytolysin are encoded respectively by $cylL_L$ and $cylL_S$, and are post-translationally modified by the *cylM* gene product. The modified cytolysin subunits are then