Tuning chemotactic responses with synthetic multivalent ligands

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Background: Multivalent ligands have been used previously to investigate the role of ligand valency and receptor clustering in eliciting biological responses. Studies of multivalent ligand function, however, have typically employed divalent ligands or ligands of undefined valency. How cells respond to multivalent ligands of distinct valencies, which can cluster a signaling receptor to different extents, has never been examined. The chemoreceptors, which mediate chemotactic responses in bacteria, are localized, and clustering has been proposed to play a role in their function. Using multivalent ligands directed at the chemoreceptors, we hypothesized that we could exploit ligand valency to control receptor occupation and clustering and, ultimately, the cellular response.

Results: To investigate the effects of ligand valency on the bacterial chemotactic response, we generated a series of linear multivalent arrays with distinct valencies by ring-opening metathesis polymerization. We report that these synthetic ligands elicit bacterial chemotaxis in both *Escherichia coli* and *Bacillus subtilis*. The chemotactic response depended on the valency of the ligand; the response of the bacteria can be altered by varying chemoattractant ligand valency. Significantly, these differences in chemotactic responses were related to the ability of the multivalent ligands to cluster chemoreceptors at the plasma membrane.

Conclusions: Our results demonstrate that ligand valency can be used to tune the chemotactic responses of bacteria. This mode of regulation may arise from changes in receptor occupation or changes in receptor clustering or both. Our data implicate changes in receptor clustering as one important mechanism for altering cellular responses. Given the diverse events modulated by changes in the spatial proximity of cell surface receptors, our results suggest a general strategy for tuning biological responses.

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Introduction

Ligand valency (the number of biologically active epitopes a multivalent ligand presents) has been shown to be important for regulating many biological processes [1,2]. In systems ranging from growth factor receptor-mediated signaling to cellular recognition, increasing the valency of the ligand increases the resultant biological response [3–12]. The increased activities of multivalent over monovalent ligands have been attributed, in some cases, to the ability of the multivalent ligand to cluster cell surface receptors, thereby initiating downstream signal transduction responses [4,13–16]. Multivalent ligands can form simultaneous contacts with multiple receptors, forcing these receptors into proximity. Clusters of certain receptors have been shown to be highly active in transducing signals due to organization of cytoplasmic components [6,16] and favorable receptor–receptor interactions [4,13]. Moreover, ligands of different valencies could result in varying levels of receptor occupation. We hypothesized that the valency of a ligand could be used to modulate cellular responses.

To explore the role of ligand valency in regulating cellular responses, a method of generating defined multivalent ligands is required. Defined molecules that have been used to investigate multivalent events are typically divalent, such as antibodies and, more recently, low molecular weight synthetic ligands [17–19]. Yet, many interesting biological systems exploit multivalent interactions of higher order valencies [1]. The generation of defined ligands of a wide range of valencies would facilitate the systematic study of multivalent interactions. Synthetic methods can be used to add epitopes to a preformed carrier, as is done commonly with bovine serum albumin–hapten conjugates [20]. Multivalent receptor–ligand interactions are complex, however, and parameters such as ligand density, orientation and spacing may also modulate the response of receptors to ligand binding. With available carrier conjugates, the effects of changes in ligand valency cannot be easily dissected from those due to changes in epitope presentation (i.e. residue density and spacing). Ideally, the chosen synthetic method should allow the variation of individual parameters. Linear synthetic multivalent scaffolds, such as acrylamide polymers or polylysine, are uniform in

presentation but are not of defined lengths [21,22]; consequently, it is difficult to ascribe the responses they elicit to specific changes in valency. Ring-opening metathesis polymerization (ROMP) can be used to create linear multivalent materials of distinct lengths with pendant polar, biologically relevant recognition epitopes [23–26]. This method has previously been used to create multivalent inhibitors of cell function [27,28]. ROMP-derived materials possess characteristics suitable for the creation of molecules to explore the relationship between ligand valency and cellular responses [22]. To investigate whether ligand valency can be used to tune a biological response, we examined the abilities of galactose-bearing polymers of differing valencies generated by ROMP to promote bacterial chemotaxis.

The molecular events leading to bacterial chemotaxis have been well studied, and the process has served as a general model for receptor-mediated responses [29–32]. During chemotaxis in *Escherichia coli*, chemoattractants, such as sugars and amino acids, and chemorepellents are recognized by specific receptors at the bacterial plasma membrane [33]. For our investigations of multivalent ligand activity, we selected galactose as a chemoattractant. The related compound, β-methyl galactopyranoside, is also a chemoattractant, indicating that the attachment of substituents at the anomeric position of galactose does not abolish its chemotactic activity [34]. This observation suggests that galactose residues could be tethered through an anomeric linker to create a multivalent display. For galactose-mediated signaling, the saccharide must bind to the soluble periplasmic glucose/galactose-binding protein (GGBP), which, in turn, interacts with the galactose-sensing chemoreceptor, Trg [34,35]. Galactose-GGBP binding to Trg initiates a signaling pathway that results in reversal of the direction of flagellar spin allowing the bacteria to swim towards the nutrient [29,30,36].

Bacterial chemotaxis requires an extremely sensitive sensing system with a broad dynamic range. Through their chemoreceptors, bacteria can detect very small changes in ligand concentration over many orders of magnitude [37,38]. A recent mathematical model proposed by Bray *et al.* to explain this remarkable feature suggests that signal transduction is regulated by changes in lateral clustering of the chemoreceptors [39–41]. In this model, clusters of bacterial chemoreceptors exchange ligand binding information, such that receptor clusters are more active in signal generation than individual receptors [39,41]. We hypothesized that ligands with distinct valencies would be able to differentially cluster the receptors and that this alteration in receptor clustering would influence the chemotactic response: larger clusters should give rise to larger signals. Here, we report that ligand valency can be used to tune the chemotactic responses of both *E. coli* and *Bacillus subtilis*. Further, we provide evidence that multivalent ligands of different valencies differentially cluster the bacterial chemoreceptors. Our results suggest that the output response due to receptor engagement is modulated, in part, by receptor clustering.

Results and discussion

ROMP-derived polymers can induce chemotactic responses

Galactose-bearing ligands of varying valencies were generated using ROMP (Figure 1). The galactose residues in the multivalent ligands are tethered to the backbone via a short linker. The attachment of this linker did not prevent binding to purified GGBP, as the interaction of monomer **1** was at least as favorable as that of galactose in an *in vitro* binding assay (data not shown). It was important, however, to determine whether galactose presented in this manner would serve as an attractant *in vivo*.

We first sought to determine whether functionalized galactose derivatives, such as monovalent **1** and multivalent **3**, possess chemotactic activity by monitoring the behavioral response of *E. coli* to these ligands. The locomotion behavior of *E. coli* occurs in two modes, running and tumbling, which are defined by the direction of the flagellar spin and, ultimately, the signal transduction response that arises from interaction of the chemoreceptor with the ligand [42]. Bacteria in the presence of an attractant will undergo prolonged running responses with low tumbling frequency [42,43]. To observe the effects of synthetic ligands on tumbling frequency, *E. coli* were treated with galactose or galactose-bearing ligands, and bacterial motion was recorded and analyzed using the method of Sager *et al.* [44]. The tumbling frequency was assessed by averaging the mean angular velocity of the paths obtained in the first

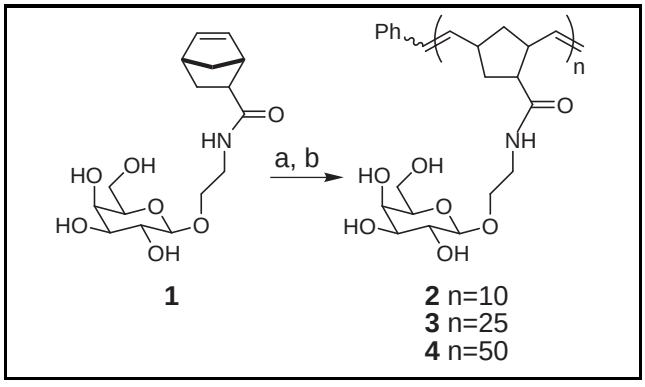

Figure 1. Structure of the galactose monomer **1** and the ROMP-derived multivalent arrays displaying galactose **2**–**4**. Emulsion polymerization conditions (**(a)** $[Cy_3P]_2Cl_2Ru=CHPh$, dodecyltrimethylammonium bromide, water, 1,2-dichloroethane, heat; **(b)** ethyl vinyl ether) were used to convert **1** to the series of oligomers **2**–**4** [24,55]. The valency (n) is calculated as described in the Materials and methods section.

5–15 s after addition of attractant (Figure 2). As expected, when bacteria were treated with increasing concentrations of galactose, the mean angular velocity decreased, an indication of a running response. Treatment with monovalent compound **1** produced similar effects, indicating that the anomeric substituent in **1** did not preclude chemotactic activity. Multivalent compound **3** was more active than monovalent **1** or unmodified galactose. It induced a low mean angular velocity even at very low (e.g. 0.001 mM) galactose residue concentrations. (For multivalent ligands, we used the galactose residue concentration not the molar concentration of ligand for comparisons.) The response of the bacteria to **3** at 0.01 mM galactose residue concentration was comparable to that obtained at a 100-fold higher (1 mM) concentration of monomer **1**. Significantly, these differences in concentration of maximum activity between the monomer **1** and multivalent **3** provided key evidence that ligand valency affects chemotactic activity.

The valency of the ligand determines the chemotactic response

To further explore the relationship between ligand valency and chemotactic response, *E. coli* were subjected to concentration gradients of compounds **1–4** in capillary accumulation assays [45]. In this procedure, attractants are evaluated by determining the concentration at which the maximum chemotactic response is achieved and the number of bacteria that accumulate at this maximum [34]. We hypothesized that increasing ligand valency in these assays may increase the potency of the response.

When compounds **1–4** were used as attractants in the capillary accumulation assay, oligomer **2** was no more active than monovalent **1**; both elicited a maximum chemotactic response at 1 mM (Figure 3a). The activity of these compounds was specific to the pendant saccharide residue, as compound **1** was not a chemoattractant for *ggbp* (AW550 and AW543) or *trg* (AW701) *E. coli* mutants (Figure 4).

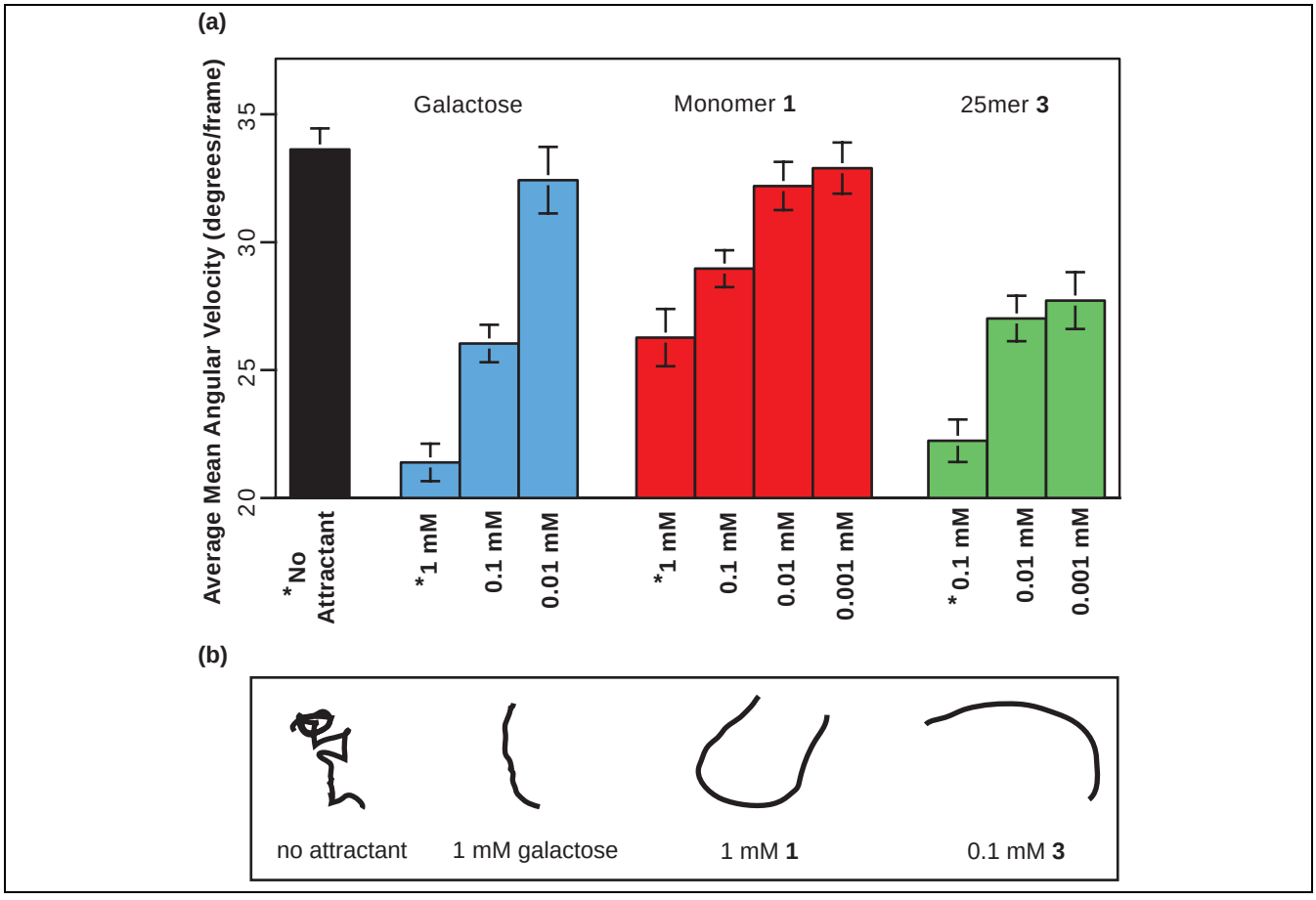

Figure 2. Results of video microscopy motion analysis experiments. (a) Bacteria were treated with buffer alone, galactose, or compound **1** or **3**, at the indicated saccharide concentrations. The results represent the average from at least five independent experiments performed in triplicate. Error bars represent the deviation between per-second averages during the 10 s interval (see Materials and methods section). Asterisks represent data for which representative video documentation is supplied (http://www.chem.wisc.edu/–kiessling/bact_vid). **(b)** Selected sample paths for bacteria treated with buffer alone, galactose, or compound **1** or **3**. Sample paths are derived from the motion of representative bacterium from a population treated with buffer alone, 1 mM galactose, 1 mM **1** or 0.1 mM **3**.

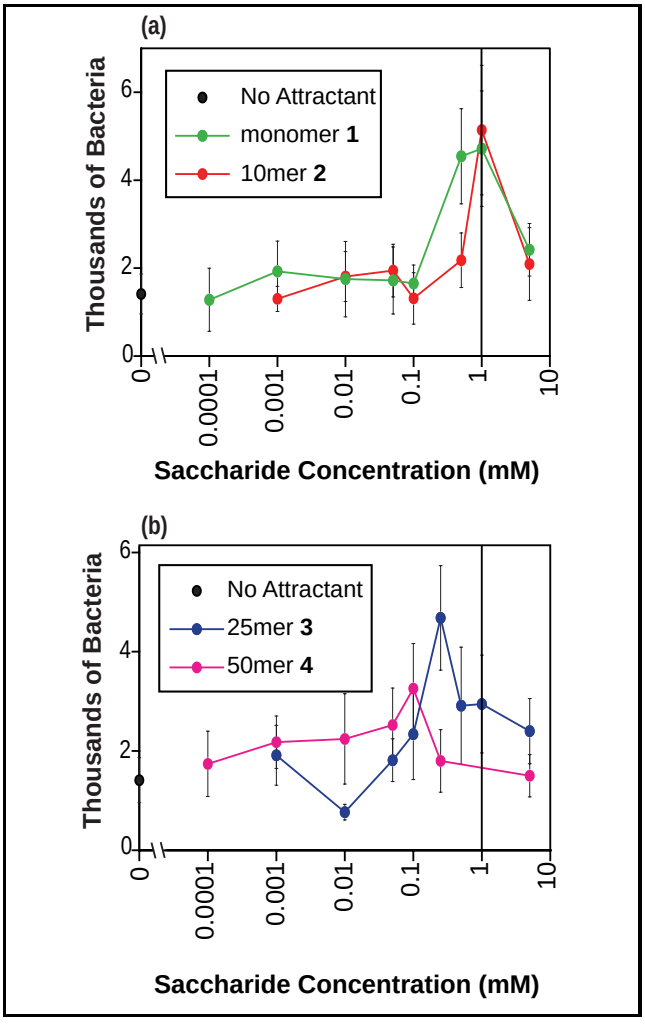

Figure 3. Results of *E. coli* capillary accumulation assays. The number of bacteria accumulated is plotted versus the concentration of the attractant calculated on a saccharide residue basis. **(a)** Results are shown for capillaries filled with buffer alone (black), **1** (green) and **2** (red) or **(b)** buffer alone (black), **3** (blue) and **4** (pink) at the indicated concentrations. The thin vertical line at 1 mM indicates the concentration of maximum chemotaxis for the monomeric compound **1**. The concentrations used in this assay are not directly comparable to those used in the motion analysis experiments (see Figure 2), because the gradient formed in the capillary assay is not defined. Results are the average of three to six experiments performed in duplicate and error bars represent a single standard deviation. Partial permeabilization was required to obtain chemotaxis towards **4** and was utilized for all experiments [57].

These results indicate that the ligands act specifically through the galactose-sensing machinery. The similarity of bacterial responses to **1** and **2** can be rationalized by a comparison of their lengths and binding capabilities. Molecular modeling studies indicate that the maximum length of oligomer **2** is approximately 50 Å [23]; consequently, it would be unlikely that this agent could cluster the chemotactic receptors, which have been found to be approximately 90 Å apart [46]. Compound **2** displays a higher local concentration of galactose to the receptor, however, the similarity in activities of **1** and **2** indicates that a high local concentration of attractant does not give rise to increased chemotactic activity. In contrast, the longer oligomers **3** and **4**, predicted by molecular modeling to be capable of clustering the receptors, were more potent. The concentrations of maximum chemotaxis were lower; the maximum for **3** is at a galactose residue concentration of 0.25 mM and the maximum for **4** is at a galactose residue concentration of 0.10 mM (Figure 3b). The ligands of higher valency (**3** and **4**), therefore, induce chemotaxis at low concentrations. The systematic shifts in the concentrations of maximum chemotaxis indicate that bacterial response can be altered in a predictable manner simply by changing the mode of ligand presentation.

The number of *E. coli* accumulated in assays employing **1**–**4** (see Figure 3) is less than that when galactose is used as an attractant (120,000 bacteria [34]), despite the observed potency of these ligands in the video assays (see Figure 2). Capillary accumulation assays depend on proper bacterial reorientations to travel into the capillary for collection. The potency of these ligands may disrupt the ability of bacteria to reorient, decreasing the apparent number of bacteria accumulated. At a given saccharide residue concentration of a multivalent ligand, fewer molecules are present to activate the receptors, and these molecules must traverse the outer membrane. These features of the system may

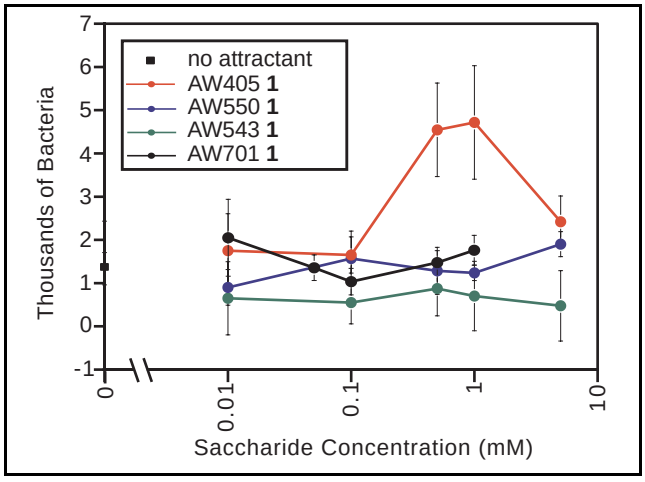

Figure 4. Disruption of galactose-sensing abrogates chemotaxis towards compound **1**. Capillary chemotaxis assays were performed using mutant *E. coli* strains AW550 (*ggbp*, blue), AW545 (*ggbp*, green) and AW701 (*trg*, black). Compound **1** was used as the attractant. Results obtained with chemotactically wild-type *E. coli* strain AW405 are shown for comparison (red). Results are the average of at least five trials performed in duplicate and error bars represent single standard deviations.

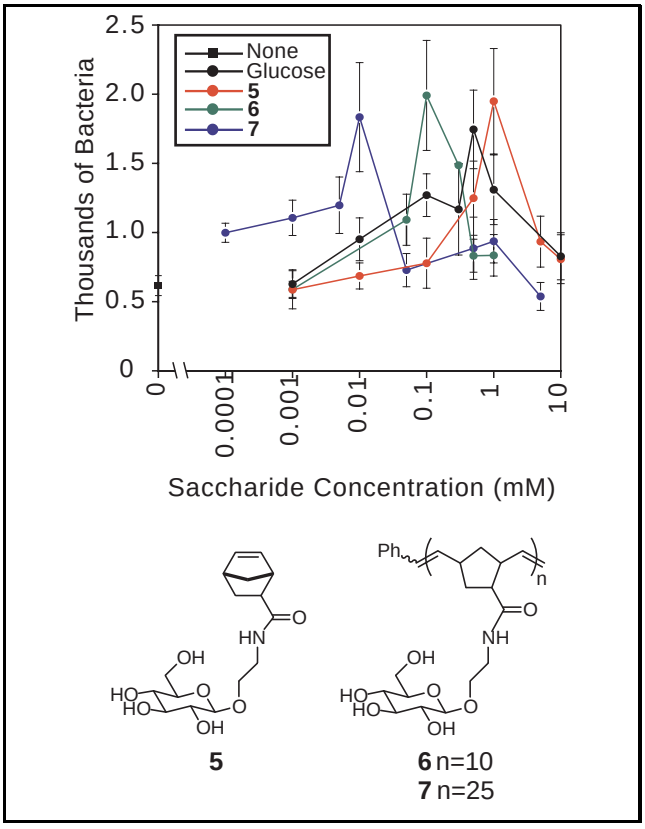

Figure 5. Results of *B. subtilis* capillary accumulation assays using ROMP-derived glucose ligands. Buffer alone, glucose or glucose-bearing compounds **5–7** were used as attractants in the capillary accumulation assay. Results are shown for glucose (black circles), **5** (red), **6** (green) and **7** (blue). Chemical structures of the attractants are shown. Results are the average of at least four trials performed in duplicate and error bars represent single standard deviations.

also contribute to the decreased numbers of bacteria accumulated.

To test the generality of the observed valency-dependent differences in chemotactic activities and to investigate the role of membrane permeability in responses to our ligands, we conducted chemotactic experiments in *B. subtilis*. *B. subtilis* is a Gram-positive bacterium that, like Gram-negative *E. coli*, is able to respond to saccharide chemoattractants [47,48]. In the case of *B. subtilis*, the multivalent ligands can directly interact with saccharide-sensing receptors, without having to first traverse the outer membrane. *B. subtilis* does not respond to galactose, but glucose is a chemoattractant for these bacteria [47]. When glucose-bearing compounds were introduced to *B. subtilis* in the capillary accumulation assay, they were effective chemoattractants. The chemotactic responses to ligands were shown to depend on ligand valency: monomer **5** elicited maximum activity at 1 mM, oligomer **6** at a saccharide residue concentration of 0.1 mM (10-fold more active than monomer **5**) and oligomer **7** at a saccharide residue concentration of 0.01 mM (100-fold more active than monomer **5**; Figure 5). Glucose had a maximal activity at 0.5 mM. In analogy to our observations with *E. coli*, as the valency of the ligand increases, the saccharide residue concentration of maximum chemotaxis decreases. Significantly, the number of bacteria accumulated towards **5–7** was comparable to the number accumulated when unmodified glucose was used as the attractant. Consistent with previous reports on the activity of galactose, galactose-bearing ligands (such as **1**) were not chemoattractants for *B. subtilis* (data not shown) [47], further suggesting that the ROMP-derived ligands were acting specifically. Together, our results indicate that in evolutionarily divergent *E. coli* and *B. subtilis* the valency of the attractant can influence chemotactic responses.

Multivalent compounds can cluster bacterial chemoreceptors

To determine directly whether multivalent ligands can influence chemoreceptor clustering, fluorescence microscopy experiments were performed to visualize changes in receptor organization upon treatment with ligand. It has been shown that wild-type *E. coli* localize chemoreceptors to their poles and that inactivation of the structural protein, CheW, results in a random distribution of chemoreceptors on the cell [49]. Using *cheW* mutants, we studied the ability of ROMP-derived arrays to localize the chemoreceptors. Bacteria were treated with **1**, **3** or **4**, fixed and labeled with an antibody to the bacterial chemoreceptors (anti-Tsr). Monovalent compound **1** had no effect on receptor distribution, but multivalent compounds **3** and **4** were observed to cluster the chemoreceptors (Figure 6). As anticipated, receptor clusters in the *cheW* cells occurred at seemingly random locations, in contrast to the polar localization observed in the wild-type bacteria. Receptor clustering was more pronounced in the case of cells treated with the longer oligomer **4** than with **3**, a result that is consistent with the differences in chemotactic activities of the ligands. The results of these experiments indicate that ROMP-derived multivalent compounds can induce lateral reorganization via receptor clustering. In addition, they suggest that changes in clustering can give rise to changes in chemotactic responses.

To ascertain whether the multivalent ligands were directly altering the clustering of the chemoreceptors in the bacterial membrane, we generated compound **8**, a galactose-bearing multivalent ligand equipped with a fluorescent label (Figure 7). When *E. coli* were treated with either fluorescein-labeled anti-Tsr antibody or with **8**, the fluorescence patterns observed were similar (Figure 8a,b). Both materials bound at the poles of the bacteria (Figure 8c). These results indicate that ROMP-derived ligands bind specifically to the bacterial chemoreceptors. To address directly the ability of these multivalent ligands to cluster receptors,

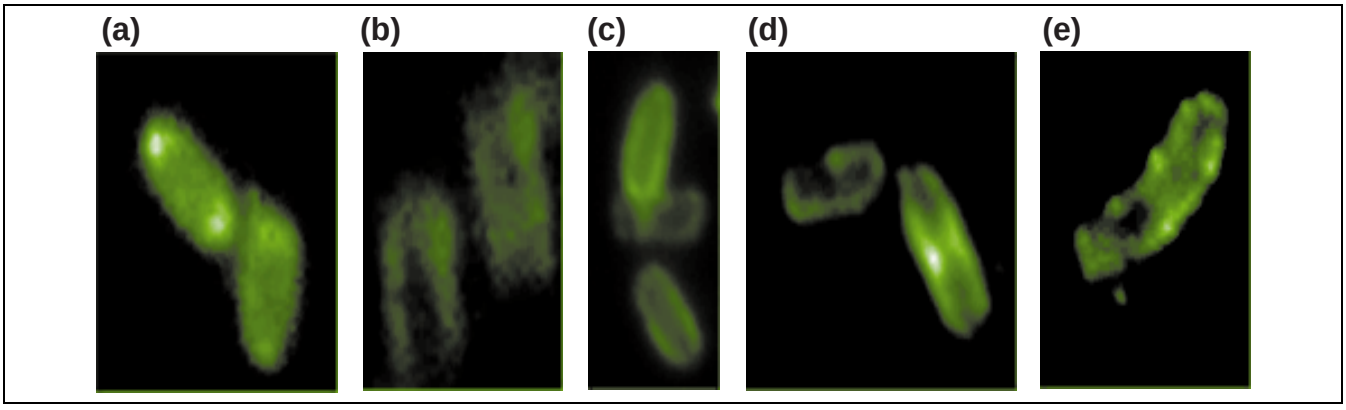

Figure 6. Visualization of receptor reorganization by ROMP-derived arrays. *E. coli* AW405 **(a)** or RP1078 (*cheW*, **b**) were pretreated with buffer alone, and *E. coli* RP1078 were pretreated with compounds **1 (c)**, **3 (d)** or **4 (e)** at 5 mM [49]. Images shown are representative of bacteria from at least two independent experiments.

we treated *CheW* mutants with both compounds. We observed patches of anti-Tsr antibody labeled chemoreceptors that colocalize with compound **8**. This result suggests that multivalent ligand **8** is responsible for the observed changes in cell surface receptor organization (Figure 8d).

Our data suggest that multivalent ligands can influence chemotactic responses by altering the extent of cell surface chemoreceptor clustering. An alternative possibility is that the multivalent ligands exhibit increases in functional affinity that lead to changes in the length of time a receptor is occupied and/or the number of receptors that are engaged. These mechanisms may both make a contribution, yet evidence linking changes in ligand affinities with chemotactic activity is lacking. Equilibrium binding constants for various ligands often do not correlate with ligand activities in bacterial chemotaxis assays [34,35,50,51]. In contrast, a number of studies have implicated receptor clustering in chemotaxis [38–41,46,52]. It has been shown that assembled tetramers of the chemoreceptor Tar are more active in *in vitro* signaling than are individual receptors or dimers [53]. Together, our data and these results suggest that the differences in chemotactic activities for monovalent **1** versus multivalent **3** and **4** are due to their abilities to change the valency of receptor clusters. We propose a mechanism in which systematic increases in ligand valency lead to changes in chemotactic responses by the incorporation of additional receptors into clusters (Figure 9).

Significance

Ligand valency and receptor clustering are critical components of many cell surface processes. Understanding the role of these factors in the regulation of cellular response is important, both for elucidating features of physiological interactions and designing compounds to elicit desired responses. We have generated synthetic molecules by ROMP that differ only in ligand valency, a feature not previously available in multivalent ligands for studying signal transduction and its ramifications. We have shown that the valency of a ligand influences its ability to cluster the chemoreceptors and its ability to elicit a chemotactic response from those receptors. Our results suggest that multivalent ligands of distinct valency, such as those described here, can be used to tune cellular responses through changes in receptor organization. Our synthetic route to multivalent arrays is general [54]; consequently, the strategy can be extended to explore a diverse spectrum of events that are mediated by receptor clustering.

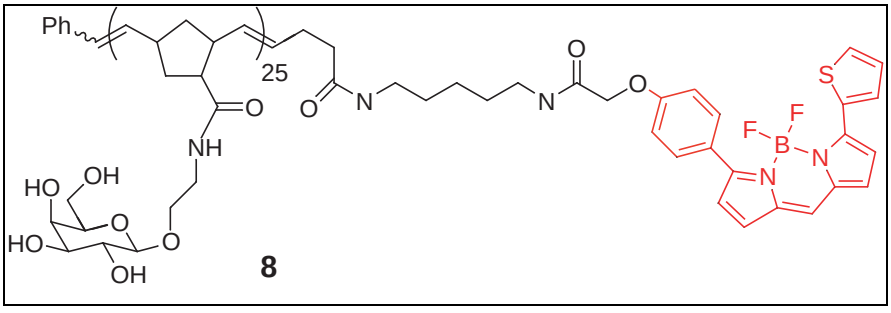

Figure 7. Structure of galactose-bearing fluorescent multivalent ligand **8**. Fluorophore (BODIPY-TR) is shown in red. The valency (n = 25) is calculated as described in the Materials and methods section.

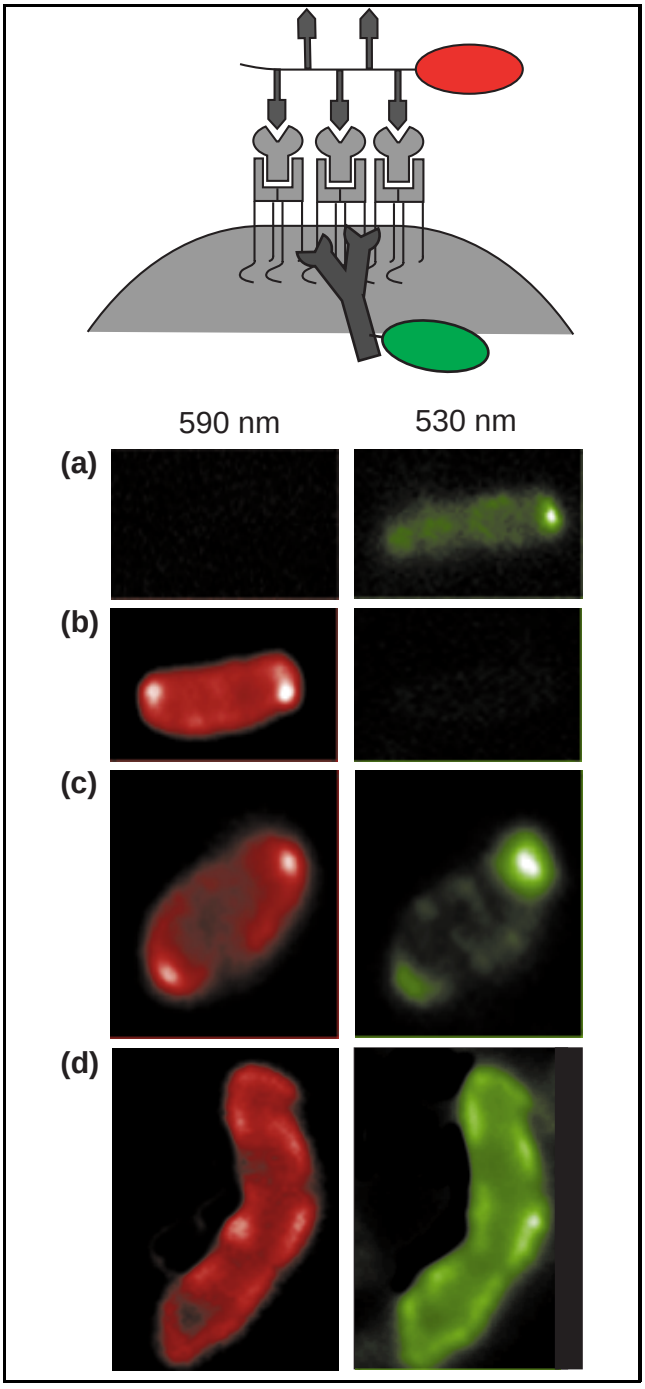

Figure 8. Multivalent ligands bind specifically to chemoreceptors and induce receptor clustering. Schematic represents fluorescently labeled **8** (red, 590 nm emission) bound to Trg via GGBP. Trg is labeled with anti-Tsr antibody (green, 530 nm emission). **(a)** *E. coli* AW405 treated with fluorescein labeled antibody as in Figure 6. **(b)** *E. coli* AW405 treated with compound **8**. **(c)** *E. coli* AW405 treated with both fluorescent antibody and **8**. **(d)** *E. coli* RP1078 (*cheW*) treated with both fluorescent antibody and **8**. Results shown are representative of those from at least two independent experiments.

Materials and methods

Generation of multivalent galactose-bearing polymers

The ROMP reaction was used to convert **1** to the series of oligomers **2**–**4** as previously described [55]. Similar conditions were employed in the synthesis of oligomers **6** and **7** [54]. Fluorescent polymer **8** was generated by specific end-labeling with a bifunctional capping agent and subsequent conjugation to the fluorophore BODIPY-TR [56]. The details of the synthesis of the capping agent will appear elsewhere. Valency (n) is an approximation of the polymer length derived from the ratio of monomer to catalyst used in the ROMP reaction.

Video microscopy

E. coli AW405, which exhibit wild-type chemotactic responses, from an overnight culture were grown in LB (Luria–Bertani broth) to an OD_{550} of 0.4–0.6 and then washed twice with attractant free chemotaxis buffer (10 mM potassium phosphate buffer, pH 7.0, 10 μM EDTA). Partially permeabilized bacteria (25 μM EDTA for 3 min at room temperature, then quench with 50 μM $CaCl_2$; this treatment had no effect on bacterial chemotaxis toward galactose or **1** but was necessary for chemotaxis toward **4**, data not shown) [57] at an OD_{550} of 0.1 were placed under a cover slip supported by additional cover slips in the method of Sager *et al.* [44]. Bacteria were allowed to adjust to contact with the glass surface for 1–2 min. The attractant was added to achieve the final concentration indicated at a 5 μl final volume. The bacterial motion at 28°C was recorded, and the paths were analyzed using the ExpertVision system. Paths derived from the first 5–15 s following the introduction of attractant were analyzed. Angular mean velocities varied approximately 14% between experiments performed on different days. Data were analyzed using the Q and Students tests.

Capillary accumulation assay

E. coli from an overnight culture were grown in LB to an OD_{550} of 0.4–0.6, washed twice with *E. coli* chemotaxis buffer and then partially permeabilized. Bacteria were resuspended in chemotaxis buffer to an OD_{550} of 0.1 and utilized in the capillary accumulation assay at 30°C for 60 min, as previously described [45]. *B. subtilis* OI1085 were grown from an overnight culture in T broth (1% tryptone, 0.2 mM $MgCl_2$, 0.5% NaCl, 0.01 mM $MnCl_2$) supplemented with 10 mM glucose and 0.5% glycerol, washed with *B. subtilis* chemotaxis buffer (10 mM phosphate buffer, pH 7.0, 10 μM EDTA, 0.5% glycerol, 0.3 mM $(NH_4)_2SO_4$) and capillary assays were performed at a final OD_{550} of 0.01 at 37°C for 30 min [47]. The amount of *B. subtilis* accumulated was normalized to 500 bacteria accumulated towards buffer alone. The results of capillary assays can be influenced by factors other than the activity of the attractant, such as metabolism of the substrate or toxicity [45,58]. To exclude this possibility, we tested the ability of *E. coli* to utilize **1** as a sole carbon source. These experiments revealed that **1**–**4** are not toxic and that monomer **1** is not metabolized (data not shown). Data was analyzed using the Q and Students tests.

Immunofluorescence microscopy

E. coli AW405 or RP1078 (*cheW*) were pretreated with buffer alone or with compounds **1**, **3**, **4** or **8** at 5 mM in a 10 μl total volume of chemotaxis buffer. After a 10 min incubation at 30°C, the bacteria were fixed (2% paraformaldehyde in Hepes pH 7.0, 30 min, 4°C), placed on poly-L-lysine treated cover slips in the bottom of six-well plates, permeabilized with methanol and labeled with anti-Tsr antibody (1:250) and fluorescein-labeled goat-anti-rabbit antibody (1:500) according to the procedure of Maddock and Shapiro [49]. Anti-Tsr antibodies recognize the conserved chemoreceptor cytoplasmic domain and are thus cross-reactive with multiple chemoreceptors (J.S. Parkinson, personal communication). Some binding exclusion (exclusive 530 or 590 nm fluorescence at a pole) was seen in dual labeling experiments in which both antibody and **8** were used. Fluorescence microscopy was performed on a Zeiss Axioscope at 1000× using an oil immersion lens. Images were captured using IPLab Spectra 3.2 and Adobe PhotoShop 5.0.

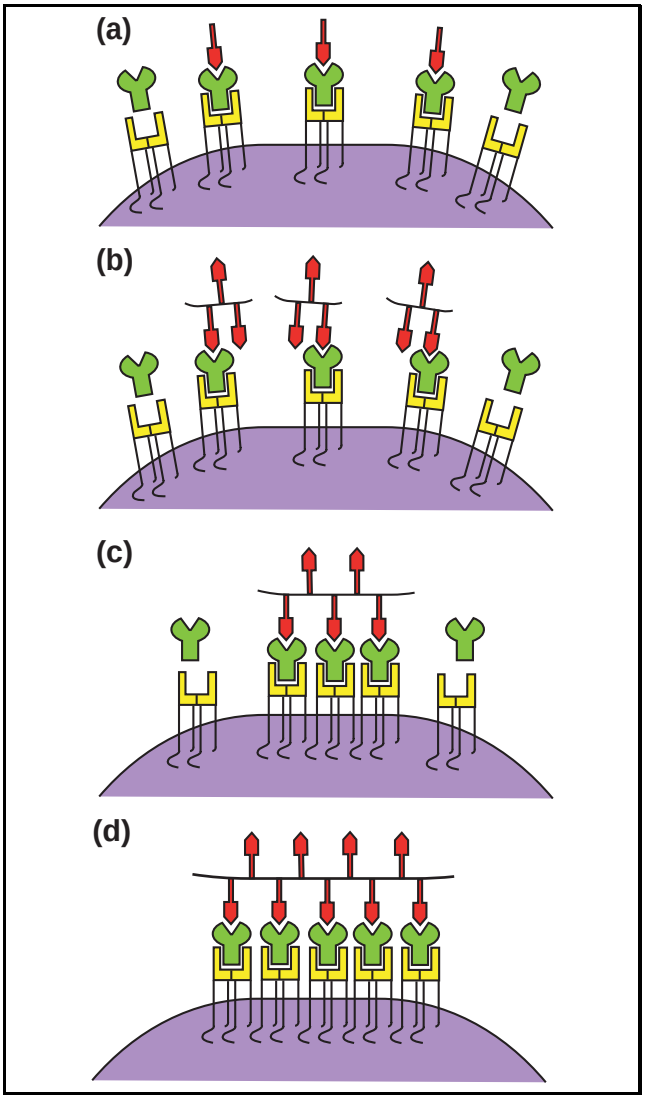

Figure 9. Model of receptor clustering by synthetic ligands. **(a)**) Chemoreceptors are observed to form dimers (or multimers) in the plasma membrane of *E. coli* and each dimer appears to interact with a single periplasmic binding protein [59,60]. Monovalent galactose ligands, such as galactose and **1**, interact with Trg through GGBP binding, inducing signal transduction from chemoreceptor dimers. **(b)** Multivalent galactose compounds, such as **2**, that cannot span the distance needed to cluster the receptors generate signals from individual dimers, as in **(a)**. **(c)** Multivalent ligands of sufficient lengths, such as **3** and **4**, are able to reorganize the chemoreceptors into discrete clusters at the plasma membrane. **(d)** Extending the valency of a multivalent ligand likely increases the size of the cluster and, therefore, the bacterial response.

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