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Abstract

Chemotaxis allows bacteria to detect specific compounds and move accordingly. This pathway involves signal detection by chemoreceptors (MCPs). Attributing a chemoreceptor to a ligand is difficult because there is a lot of redundancy in the MCPs that recognize a single ligand. We propose a methodology to define which chemoreceptors bind a given ligand. First, an MCP is overproduced to increase sensitivity to the ligand(s) it recognizes, thus promoting accumulation of cells around an agarose plug containing a low attractant concentration. Second, the ligand-binding domain (LBD) of the chemoreceptor is fused to maltose-binding protein (MBP), which facilitates purification and provides a control for a thermal shift assay (TSA). An increase in the melting temperature of the LBD in the presence of the ligand indicates that the chemoreceptor directly binds it. We showed that overexpression of two *Shewanella oneidensis* chemoreceptors (SO_0987 and SO_1056) promoted swimming toward an agarose plug containing a low concentration of chromate. The LBD of each of the two chemoreceptors was fused to MBP. A TSA revealed that only the LBD from SO_1056 had its melting temperature increased by chromate. In conclusion, we describe an efficient approach to define chemoreceptor-ligand pairs before undertaking more-sophisticated biochemical and structural studies.

Keywords: Bacterial chemotaxis; Signal detection; Methyl-accepting Chemotaxis Protein; Thermal Shift Assay; Agarose-in-plug bridge assay; MBP chimeric proteins.

1. Introduction

Bacteria encounter changing environments to which they adapt via a variety of mechanisms. In addition to adaptation through the regulation of gene expression, bacteria also control their movement within their environment using a mechanism called chemotaxis [1,2]. Upon detection of signal molecules called chemoeffectors, cells modulate their swimming behavior to move toward attractants or away from repellents. Chemotaxis is governed by a complex regulatory system composed of various Che proteins [3]. The well-conserved CheA-CheY histidine-kinase/response-regulator pair, which engage in a phosphorylation cascade, directly controls the rotational direction of the flagellum, which propels the bacterium. The first step in chemotaxis is the detection of chemoeffectors by specific chemoreceptor proteins called MCPs (Methyl-accepting Chemotaxis Proteins) [2,4]. The binding of chemoeffectors to MCPs then affects the phosphorylation cascade involving CheA-CheY, and consequently, bacterial movement.

Bacterial genomic analysis has revealed a large number of MCP-encoding genes, with some bacteria containing more than 80 [5]. Chemoreceptors are characterized by the presence of a highly conserved signaling domain within their cytoplasmic region. However, they also contain additional domains. Chemoreceptors can be classified according to the number of helical heptads that are contained within their signaling domain [5,6]. They can also be classified based on their topologies and the type of ligand-binding domain (LBD) they contain [4,7]. Cache, 4HB and PAS-containing domains are among the most common types of LBDs. The first described Cache domains were involved in small-molecule recognition [8]. Today, a large superfamily of different types of single and double Cache domains has been documented, and some ligands, such as amino acids, polyamines, carboxylic acids, sugars and hydrocarbons have been identified [9–11]. The 4HB (four-helix bundle) domain, which is widespread in signal transduction systems, has been shown to bind ligands such as amino, aromatic and carboxylic acids directly [10,12]. PAS domains, which are exclusively present in the cytoplasm, allow signal detection via direct binding to small molecules, including divalent cations or carboxylic acids. PAS-domain interactions can also be mediated by the presence of cofactors (heme b or c, FAD, FMN) for detection of signals such as oxygen or redox potential [13].

Escherichia coli, which has been a model for bacterial chemotaxis for many years, possesses only five chemoreceptors, all of which have been well characterized [14]. Four *E. coli* chemoreceptors contain a 4HB domain (Tar, Tsr, Trg and Tap), while one possesses a FAD-containing PAS domain (Aer). *E. coli* is able to respond chemotactically to various signals, including amino acids, sugars, dipeptides, quorum-sensing signals, pH and redox potential. Some signals are directly detected by the chemoreceptors, while the detection of others requires auxiliary proteins. For example, the Tar

chemoreceptor detects aspartate by direct binding to the Tar LBD and recognizes maltose via the maltose-binding protein (MBP).

Many bacteria have far more chemoreceptors than *E. coli*, especially bacteria that typically encounter changing environmental conditions [15]. For example, several species of the *Pseudomonas* genus contain more than 20 chemoreceptors. Aside from classical ligands (amino acids, carboxylic acids, sugars), some Pseudomonads use specific chemoreceptors to detect atypical molecules such as toluene, aromatic hydrocarbons and naphthalene [16]. *Shewanella oneidensis*, an aquatic bacterium, possesses 27 chemoreceptors. It is chemotactically attracted to various respiratory substrates (oxygen, nitrate, fumarate, TMAO), but, more surprisingly, it is also attracted to chromate, a toxic compound [17–19]. While five chemoreceptors have been shown to be involved in responses to respiratory substrates [18], the chemoreceptor(s) responsible for detecting chromate remain(s) unknown.

Despite the fact that the number of characterized LBDs has increased over last few years, it remains challenging to assign a ligand to a chemoreceptor, and vice versa. This is especially true because MCPs containing LBDs of the same type may detect many different ligands. Additionally, the same ligand may be detected by MCPs with different types of LBD. One strategy corresponds to the characterization of chemoreceptors one by one, testing their capacity to bind molecules from various libraries *in vitro* [20,21]. This strategy allows the identification of one or several compounds capable of binding a chemoreceptor. However, compounds identified in this way are not necessarily chemoeffectors, since the method does not assess their capacity to elicit a chemotactic response. Nevertheless, this strategy can be helpful when no chemoeffector has been identified within the organism studied, since the identification of chemoreceptor ligands has the potential to inform about the nature of compounds to which an organism may respond. However, a chemoeffector detected by a specific chemoreceptor can be missed using this strategy, because many chemoreceptors detect signals indirectly, for instance via interactions with periplasmic ligand-binding proteins.

Use of a different strategy based on gene deletions is possible when compounds are known to elicit chemotactic behavior for a given bacterium since, in this scenario, one must determine which chemoreceptor(s) is involved in the detection of a specific compound [22]. As mentioned above, this too may be complicated by the fact that bacteria usually possess many chemoreceptors and more than one chemoreceptor is often capable of detecting the same ligand. Here, we propose an easy and inexpensive strategy combining optimized versions of *in vivo* and *in vitro* experiments to determine which chemoreceptor(s) is/are involved in the direct recognition of a ligand via their LBD.

2. Materials and Methods

2.1 Strains.

The wild-type *S. oneidensis* strain used in this study was MR1-R [23]. All strains were routinely grown at 28°C (*S. oneidensis* strains) or 37°C (*E. coli* strains) in lysogeny broth (LB) medium. When necessary, chloramphenicol (25 μg/ml), rifampicin (10 μg/ml) or ampicillin (50 μg/ml) was added. The concentration of arabinose used for inducing expression of plasmid-borne genes was 0.2%.

2.2 Construction of double-deletion mutant.

The ΔSO0987ΔSO1056 mutant strain was constructed by sequential deletion of the *so_0987* and *so_1056* genes from the genome of the wild-type strain. This deletion mutant was constructed by homologous recombination as described previously [18]. Briefly, 500 bp flanking regions downstream and upstream of the gene to be deleted were cloned into the suicide vector pKNG101. The resulting plasmid was introduced into the *E. coli* strain CC118λpir [24] and transferred into the appropriate *S. oneidensis* strain by conjugation using the *E. coli* helper strain 1047/pRK2013 [25]. The plasmid was integrated into the chromosome by a first recombination event and removed by a second recombination event in the presence of 6% sucrose. Deletions were confirmed using PCR and appropriate primers.

2.3 Construction of plasmids.

All plasmids used in this study are listed in Table 1. To construct pSO0987, pSO1056, pSO2240, pSO3282, pSO4053, pSO4454 and pSO4557, the coding sequences of the respective genes were amplified by PCR using *S. oneidensis* DNA as a template and primers containing appropriate restriction sites and an optimized Shine Dalgarno sequence. After digestion, PCR products were inserted into the pBAD33 vector. To construct pMAL-LBD-0987 and pMal-LBD-1056, the sequences encoding the LBD of SO_0987 (Y30 to S200) and SO_1056 (S29 to M278) were amplified by PCR using *S. oneidensis* DNA as a template and primers containing appropriate restriction sites. After digestion, PCR products were inserted into the pMAL-c2X vector. The sequences of all constructs were confirmed via DNA sequencing using appropriate primers.

Table 1

Plasmids used in this study

Plasmids	Descriptions	References
pBAD33	Vector containing pBAD promoter with the p15A origin of replication, Cm^R	[26]
pSO0987	*so_0987* sequence cloned into pBAD33	This work
pSO1056	*so_1056* sequence cloned into pBAD33	This work
pSO2240	*so_2240* sequence cloned into pBAD33	This work
pSO3282	*so_3282* sequence cloned into pBAD33	This work
pSO4053	*so_4053* sequence cloned into pBAD33	This work
pSO4454	*so_4454* sequence cloned into pBAD33	This work
pSO4557	*so_4557* sequence cloned into pBAD33	This work
pMAL-c2X	Vector containing p*tac* promoter and the coding sequence of MBP, Amp^R	New England Biolabs
pMAL-LBD-0987	Sequence coding for the ligand-binding domain of SO_0987 (Y30 to S200) cloned into pMAL-c2X	This work
pMAL-LBD-1056	Sequence coding for the ligand-binding domain of SO_1056 (S29 to M278) cloned into pMAL-c2X	This work
pKNG101	R6K-derived suicide plasmid containing Str^R and *sacB*	[24]
pRK2013	RK2-Tra1 RK2-Mob1 Kan^R *ori* ColE1	[25]

2.4 Agarose-in-plug bridge assays.

2.4.1 Construction of bridge slide. Microscope bridge slides were constructed as described previously [27]. Briefly, two square coverslips were placed on each side of a PETG slide. Four to eight agarose plugs per slide were made by pipetting 5µl preheated 2% low-melting agarose that contained the chemical to be tested onto the slide. Within a few seconds, a glass coverslip (24 mm × 60 mm) was placed over the slide. The bridge structure is maintained using a cyanoacrylate adhesive.

2.4.2 Cell preparation and microscopic observation. Cells were prepared as described in Armitano et al. [27], with slight modifications. Briefly, cells were grown overnight in LB medium under shaking conditions at 28°C. Cells were subsequently diluted in fresh LB medium at an OD_{600} value of 0.05 and grown under shaking conditions until an OD_{600} value of 0.7-0.8 was reached. Cells were then harvested by centrifugation (10 min at 3,500 rpm) and gently resuspended in LM medium (0.2 g/l yeast extract, 0.1 g/l peptone, 10 mM HEPES (pH 7.4) and 10 mM NaHCO3) containing 20 mM lactate and 0.1% Tween-20 to produce an OD_{600} of 0.2. After 30 min of static incubation at room temperature, 150 µL of cell suspension was introduced within the microscope slide bridge. Photographs were taken at the edges of the plugs after 30 min with a 4X phase contrast objective. Strains containing pBAD33, pSO0987, pSO1056, pSO2240, pSO3282, pSO4053, pSO4454 or pSO4557 were grown overnight in the presence of chloramphenicol, and arabinose was subsequently added to induce gene expression.

2.5 Expression and purification of MBP-LBD chimeric proteins.

Recombinant MBP-tagged proteins, MBP-LBD_{0987} and MBP-LBD_{1056}, were produced from the *E. coli* C600 strain [28] that contained a pMAL-LBD-0987 or pMAL-LBD-1056 plasmid, respectively. The strains were grown aerobically at 37°C in LB medium. At OD_{600} = 0.5, overproduction of proteins was induced with 0.3 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 3 h at 37°C, cells were collected by centrifugation and resuspended in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM EDTA. Thereafter, they were lysed using a French press and centrifuged at 11,000 rpm for 20 min at 4°C. After an ultracentrifugation step at 45,000 rpm for 1 h at 4°C, the supernatant was loaded onto an amylose high-flow resin (New England Biolabs), and proteins were purified according to the manufacturer's protocol. Purified proteins were loaded onto a PD-10 desalting column (GE Healthcare) and recovered in 20 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. Protein

concentrations were determined using a Bradford assay (Bio-Rad). Purified proteins were store at - 80°C in 20 mM Tris-HCl (pH 7.4), 100 mM NaCl and 10% glycerol.

2.6 Thermal shift assays.

TSAs were carried out using purified proteins in 20 mM Tris-HCl (pH 7.4), 100 mM NaCl and 10 % glycerol in a total volume of 20 µL, as described previously [29]. Briefly, either MBP-LBD_{0987} (11.2 µM) or MBP-LBD_{1056} (7.8 µM) was incubated in the presence of 10X SYPRO Orange (Sigma Life Science) with or without potassium chromate (1 mM and 10 mM) and maltose (10 mM). Samples were then heated from 10 to 90°C at a scan rate of 0.5°C per 30 s using a BioRad CFX96 Touch Real Time PCR instrument. Protein unfolding curves were monitored by detecting changes in SYPRO Orange fluorescence. Melting temperatures were determined from first derivative values of raw fluorescence data using BioRad CFX Manager 3.1 software.

3. Results and Discussion

3.1 Scientific rationale of the experimental strategy.

Various techniques have been described to test the chemotactic abilities of bacteria. Among these, the agarose-in-plug bridge assay has been successfully used to study the behavior of different bacterial species [16,27,30–34]. Using this approach, the chemical to be tested is included in an agarose drop and placed on a microscope slide. A bacterial suspension is then injected in the area surrounding the agarose. If the chemical is an attractant, its diffusion into the bacterial suspension will cause cells to move toward the plug, which produces a microscopically observable bright ring of cells. One can then compare the behavior of a wild-type strain to that of mutants that harbor an MCP-deletion.

However, because signal-detection redundancy occurs, in which several MCPs bind the same ligand, the behavior of a single MCP-deletion mutant may not be distinguishable from that of its wild-type counterpart. Moreover, a gene-deletion analysis is not an easy task for bacteria that contain many chemoreceptor-encoding genes. To overcome this pitfall, we propose the use of a reverse methodology. Using this experimental rationale, instead of deleting an MCP-encoding gene in order to observe a reduced chemotactic response after exposure to a specific ligand, we propose cloning and overexpressing MCP-encoding genes in a wild-type strain. As the relative expression level of a chemoreceptor defines the sensitivity of response it mediates [35], the capacity of the resulting strains

to detect and respond to a concentration below that to which a control strain containing an empty vector is able to respond will be checked (Fig. 1A and B).

The chemoreceptor(s) identified by this approach will be tested *in vitro* using a simple thermal shift assay (TSA) to determine whether or not the detection of chemoattractant occurs via direct binding of the ligand to the chemoreceptor(s). Since the heterologous expression of entire chemoreceptors or of their LBDs in *Escherichia coli* could result in the formation of insoluble proteins that form inclusion bodies [36], we suggest that LBDs should be produced as chimeric proteins fused to MBP. Fusion to MBP has previously been shown to facilitate the solubilization of proteins that otherwise formed inclusion bodies (Fig. 1C) [37,38]. This strategy is an alternative to the recovery of proteins from inclusion bodies, which requires subsequently refolding and purification [36]. Interaction between a chimeric protein and ligand can then be assessed using a TSA [39]. This rapid and inexpensive technique is easy to set up and requires only a thermocycler, which is commonly found in microbiology laboratories. Moreover, it requires only low concentrations of proteins and can be performed in 96-well microplates, unlike isothermal titration calorimetry (ITC), which is a direct binding test commonly used to confirm ligand-protein interactions.

TSA requires proteins to be subjected to thermal denaturation in the presence of a dye (SYPRO Orange), which is fluorescently active when bound to hydrophobic domains of proteins exposed by thermal unfolding. Usually, ligand binding to a protein increases its thermal stability, which produces a detectable shift to a higher melting temperature (Fig. 1D). Moreover, TSA was previously proved to be a suitable technique for ligand-chemoreceptor interaction screening [20,21,40,41]. Adding MBP to form a chimeric protein provides an additional advantage, since MBP is able to function as an internal control whose denaturation profile should only be affected by maltose, its natural ligand. Below, we describe how this combined strategy was used to characterize chemoreceptor(s) involved in the detection of chromate, an attractant peculiar to *S. oneidensis*.

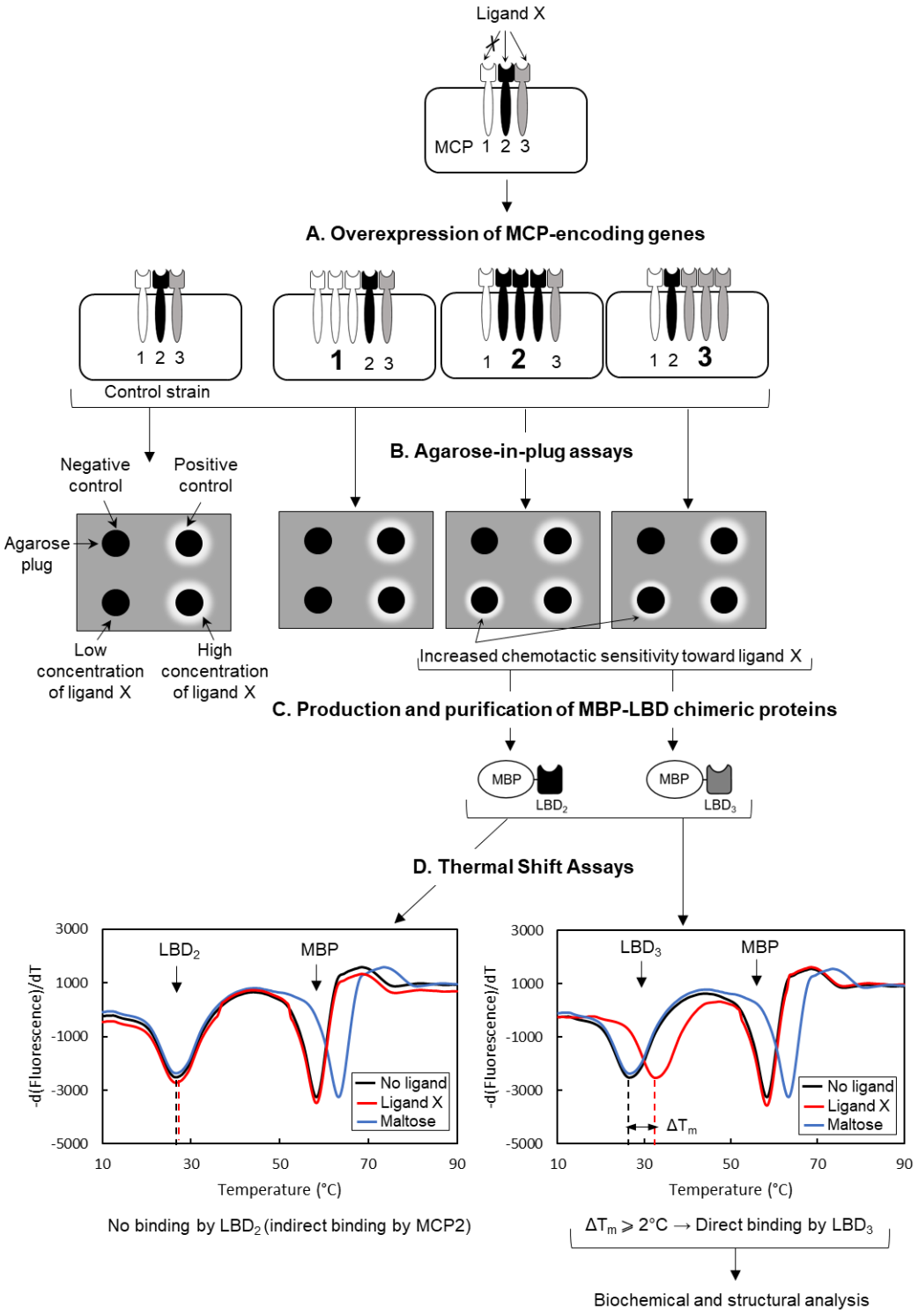


Figure 1. A scheme outlining the strategy used to identify chemoreceptors (MCPs) involved in specific ligand recognition. A) Each gene encoding an MCP to be tested was overexpressed in a wild-type background. B) Agarose-in plug assays were used to test the chemotactic behavior of each MCP-overexpressing strain toward ligand X. MCPs whose overproduction enhances the chemotactic sensitivity of the wild-type strain, as indicated by a bright ring of cells around a plug containing a low concentration of ligand X, are selected as candidates for ligand X recognition. C) The ligand-binding domains (LBD) of the selected MCPs are fused to maltose-binding protein (MBP). The resulting MBP-LBD fusions are overproduced and purified, taking advantage of the ability of MBP to bind to amylose. D) Interactions between MBP-LBD

fusions and ligand X are tested using TSA. Curves of the first derivative of fluorescence emission with respect to temperature should contain two negative peaks that corresponded either to the MBP or LBD moiety of each chimeric protein. Maltose is used to identify the peak that corresponds to MBP. The two peaks allow us to determine the melting temperature (T_m) of each moiety of the chimeric protein in the absence and presence of ligand X. When the addition of ligand X does not modify the T_m of the LBD, ligand X probably does not bind directly to the LBD. A T_m shift for the LBD in the presence of ligand X ($\Delta T_m \geqslant 2°C$) indicates that the LBD binds ligand X directly. The LBD-ligand X interaction can then be further characterized with other methods.

3.2 Combining the overexpression of chemoreceptor-encoding genes with an agarose-in-plug bridge assay enabled the identification of two chemoreceptors involved in the chemotactic response to chromate.

S. oneidensis is attracted to chromate, a toxic metal to which it is resistant [19]. To the best of our knowledge, this is the only example of bacterial chemotaxis toward chromate. Therefore, no specific motif for chromate binding has been previously described. Recently, we described an efflux pump, $ChrA_{SO}$, involved in *S. oneidensis* chromate resistance [42]. A gene (*so_0987*) encoding a chemoreceptor was identified immediately downstream and in the same orientation as the *$chrA_{SO}$* (*so_0986*) gene. Apart from its location, the *so_0987* gene drew our attention because it encodes a chemoreceptor with a Cache domain in its LBD. Since Cache domains are capable of binding small molecules, we decided to test not only SO_0987 but also other Cache-containing chemoreceptors for their ability to detect chromate [8]. The *S. oneidensis* genome is predicted to encode the following Cache-domain containing chemoreceptors: SO_0987 and SO_2240 (sCache2); SO_1056, SO_1278, SO_4454, SO_4557 and SO_4466 (dCache1); SO_4053 (dCache3) and SO_3282 (Cache3-Cache2) [5]. Except for SO_1278 and SO_4466, which were associated with lowest Cache domain prediction E-values, all genes encoding Cache-containing chemoreceptors were independently cloned under the control of an arabinose-inducible promoter. The resulting plasmids were introduced into the wild-type *S. oneidensis* reference strain (MR1-R), and chemotactic behaviors of the strains were tested via the agarose-in-plug bridge assay.

As shown in Figure 2A, the wild-type strain that contained an empty vector was attracted to nitrate (used as a control) and also to chromate when it was present at 10 mM in the plug, as indicated by the presence of a bright ring of cells close to the plug. However, when only 1 mM chromate was added to the plug, it did not attract wild-type bacteria. Among the seven chemoreceptor genes tested, the overexpression of only two (*so_0987* and *so_1056*) resulted in a bright ring of cells around the plug containing 1 mM chromate; attraction to nitrate remained unchanged (Fig. 2A). These results indicate

that the cells overexpressing either *so_0987* or *so_1056* are attracted to chromate with a higher sensitivity than the control strain.

In addition to these two chemoreceptors, likely others are also involved in the detection of chromate, as a strain containing deletions of *so_0987* and *so_1056* was still attracted to chromate (Fig. 2B).

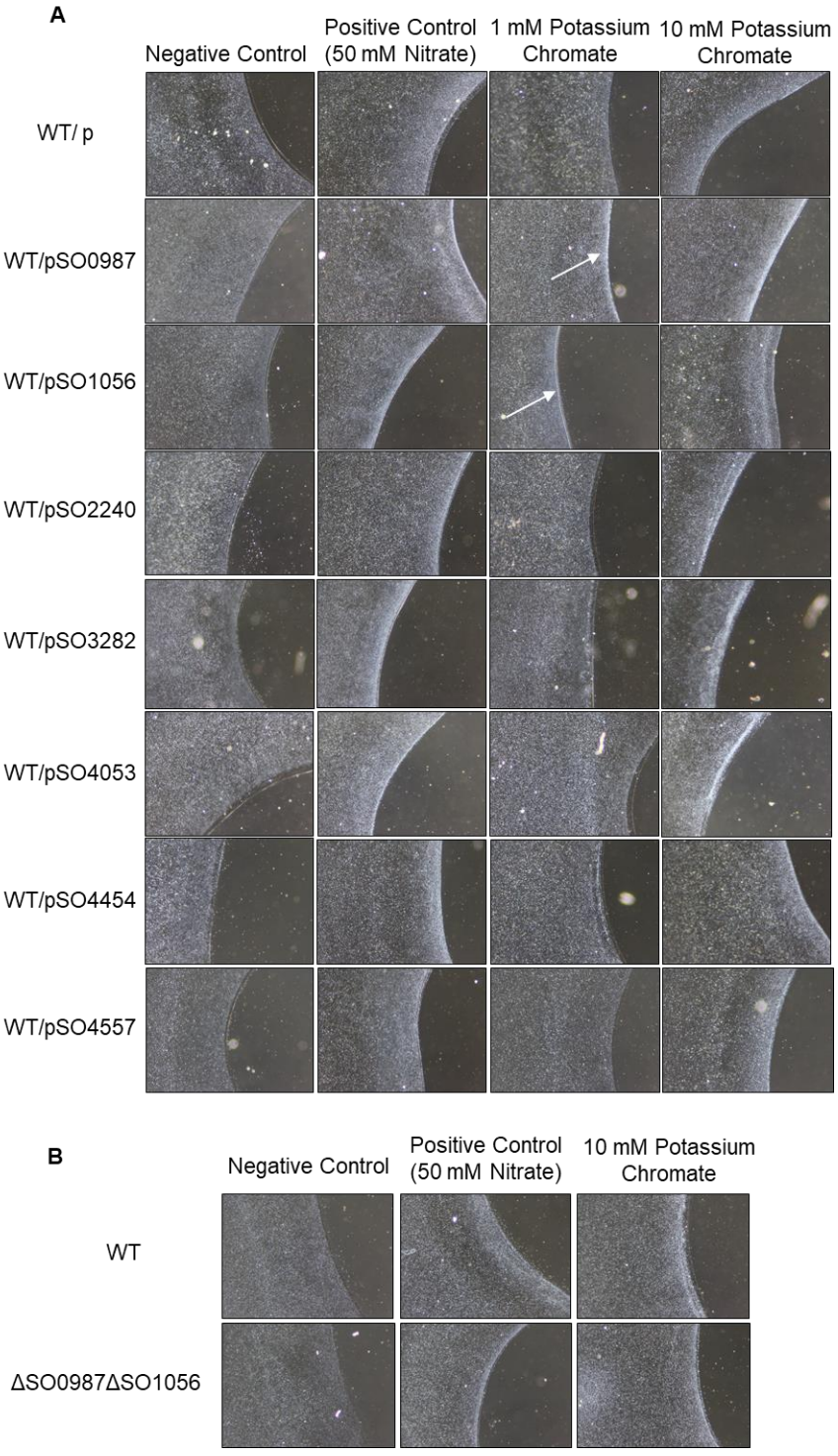


Figure 2. Agarose-in-plug bridge assays to identify dedicated chemoreceptors. A) Agarose-in-plug bridge assays of a wild-type (WT) *S. oneidensis* strain containing either an empty vector (p) or one of the seven plasmids bearing Cache-

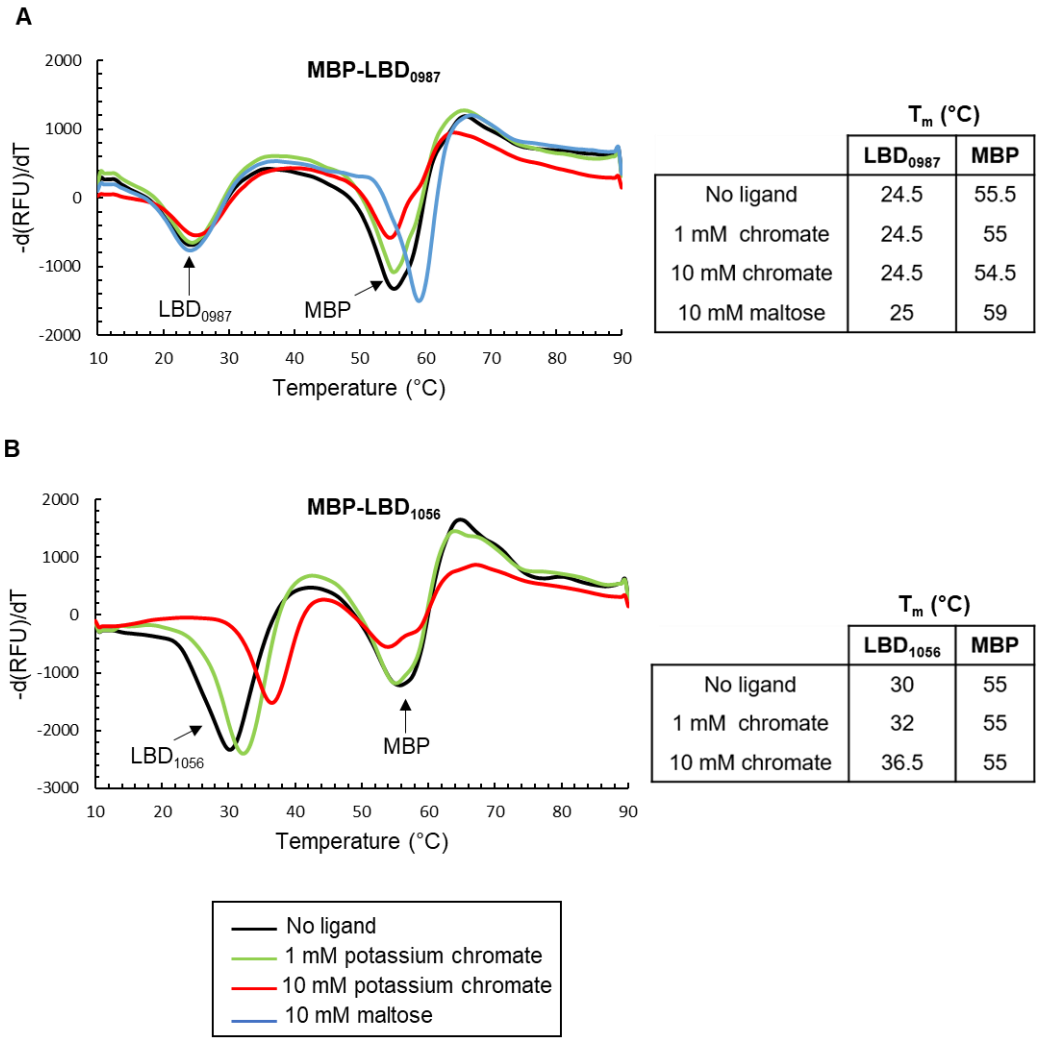
containing MCP-encoding genes (pSO0987, pSO1056, pSO2240, pSO3282, pSO4053, pSO4454 or pSO4557) are shown. B) Agarose-in-plug bridge assays of wild-type (WT) and ΔSO0987ΔSO1056 strains are shown. Cells were grown in LB containing 0.2% arabinose under shaking conditions until an OD_{600} of 0.7-0.8 was reached. Cells were then harvested by centrifugation and resuspended in LM medium containing lactate and Tween-20. The chemotactic behaviors of the strains toward a plug that contained either water (negative control), 50 mM sodium nitrate (positive control), or 1 mM or 10 mM potassium chromate were tested using an agarose-in-plug bridge assay. Photographs were taken at the edges of the plug after 30 min of incubation (4× magnification). White arrows indicate the accumulation of cells around a plug containing a low concentration of potassium chromate (A). All pictures are representative of three independent experiments.

3.3 The LBD of one of the two identified chemoreceptors directly binds chromate.

To test whether the detection of chromate by the two chemoreceptors is direct, we performed TSAs using purified proteins. The SO_0987 and SO_1056 proteins are predicted to share a similar topology, with two transmembrane segments delimiting the LBD. Each LBD is predicted to be periplasmic and contains a Cache domain. Therefore, we cloned the region encoding the LBD of each chemoreceptor in frame with an MBP-encoding gene. The gene fusions were placed under the control of the IPTG-inducible p*tac* promoter, which allowed their overexpression. The two chimeric MBP-LBD proteins proved to be soluble when overproduced. Overproduction of the corresponding Strep-tagged LBDs resulted in insoluble proteins that were found in inclusion bodies.

The two chimeric MBP-LBD proteins were subsequently purified using an amylose resin column. The two purified proteins were then submitted to thermal denaturation in the presence of SYPRO Orange fluorescent dye. To determine the melting temperature (T_m), the first derivatives of melt curves are shown (Fig. 3). Similar curves displaying two peaks were observed for each chimeric protein in the absence of ligand (Fig. 3A and B; black curves). We assumed that each moiety of the chimeric protein is capable of folding independently, and thus reacts differently to thermal denaturation.

To assign each peak to a specific moiety of the chimeric protein, we submitted the MBP-LBD_{0987} protein to thermal denaturation in the presence of maltose, the ligand of MBP. Only the second peak of the melt curve of the chimeric protein shifted in response to the presence of maltose, indicating that this peak corresponded to the MBP moiety (Fig. 3A, blue curve). This strongly suggested that the first peak was associated with the LBD moiety of the chimeric protein.



	T_m (°C)	
	LBD_{0987}	MBP
No ligand	24.5	55.5
1 mM chromate	24.5	55
10 mM chromate	24.5	54.5
10 mM maltose	25	59

	T_m (°C)	
	LBD_{1056}	MBP
No ligand	30	55
1 mM chromate	32	55
10 mM chromate	36.5	55

Figure 3. Thermal shift assays with MBP-LBD_{0987} and MBP-LBD_{1056} in the absence and presence of potassium chromate and maltose. A) Thermal shift assays with MBP-LBD_{0987} in the absence or in the presence of 1 or 10 mM potassium chromate, or 10 mM maltose, are shown. B) Thermal shift assays with MBP-LBD_{1056} in the absence or in presence of 1mM or 10 mM potassium chromate are shown. Graphs represent the first derivative of the fluorescence emission (-d(RFU)/dT, RFU: Raw Fluorescence Unit) as a function of temperature. The melting temperatures (T_m) of each peak are listed in the tables to the right of each graph. Each graph is colored according to the tested ligand, as indicated. All graphs represent the results of three independent experiments.

We then performed TSAs to test chromate binding to both chimeric proteins. For MBP-LBD_{0987}, neither 1 mM nor 10 mM chromate shifted the melt curve, and the T_m calculated for each moiety was not significantly modified (Fig. 3A). In contrast, for MBP-LBD_{1056}, chromate modified its melting curve. Only the peak assigned to the LBD_{1056} moiety was altered, whereas the peak corresponding to MBP remained unaffected (Fig. 3B), signifying that the T_m associated with the MBP moiety remained

unchanged by chromate. The T_m that corresponded to the LBD_{1056} moiety increased significantly (2°C and 6.5°C in the presence of 1 mM and 10 mM chromate, respectively). A T_m shift of 2°C or more is considered significant and is a strong indicator of ligand binding [11,21,40,41].

Of the two chemoreceptors we identified as being involved in the chemotactic response to chromate, TSA indicated that one (SO_1056) directly binds chromate via its Cache-containing LBD. For the second chemoreceptor (SO_0987), no evidence of direct binding of chromate to the LBD was obtained. This finding indicates that either the chemoreceptor binds chromate via a different region of the protein, that chromate detection is indirect and requires an additional protein, or that for some unknown reason chromate binding to the LBD does not cause a significant shift in the melting temperature.

4. Conclusion

We previously developed a high-throughput technique to identify novel molecules that elicit a chemotactic response in bacteria [19]. Here, we propose a combination of efficient and simple protocols that can be used to assign chemotaxis-promoting compounds to chemoreceptors that bind their ligands directly. This approach can easily be scaled-up to perform high-throughput screening of chemoreceptors for a given ligand. As this is only a first step towards understanding signal detection, further characterization of the identified chemoreceptors must later be performed using more thorough biochemical techniques such as microscale thermophoresis, isothermal titration calorimetry, and structural analysis. The same approach could be used to identify the precise ligand-binding region after random mutagenesis of an LBD. Finally, these combined methods should be useful for biotechnology engineering, for example to design tools to detect pollutants.

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The manuscript has been professionally proofread (Proof-Reading-Service.com).

AB, VM and CJC designed research. AB, AAC and CJC performed experiments. AB, AAC, VM and CJC analyzed data. AB, VM and CJC wrote the manuscript.

The authors declare no conflict of interest.

References

[1] G.H. Wadhams, J.P. Armitage, Making sense of it all: bacterial chemotaxis, Nat. Rev. Mol. Cell Biol. 5 (2004) 1024–1037. https://doi.org/10.1038/nrm1524.

[2] S. Bi, V. Sourjik, Stimulus sensing and signal processing in bacterial chemotaxis, Curr. Opin. Microbiol. 45 (2018) 22–29. https://doi.org/10.1016/j.mib.2018.02.002.

[3] V. Sourjik, N.S. Wingreen, Responding to chemical gradients: bacterial chemotaxis, Curr. Opin. Cell Biol. 24 (2012) 262–268. https://doi.org/10.1016/j.ceb.2011.11.008.

[4] A.I.M. Salah Ud-Din, A. Roujeinikova, Methyl-accepting chemotaxis proteins: a core sensing element in prokaryotes and archaea, Cell. Mol. Life Sci. 74 (2017) 3293–3303. https://doi.org/10.1007/s00018-017-2514-0.

[5] V.M. Gumerov, D.R. Ortega, O. Adebali, L.E. Ulrich, I.B. Zhulin, MiST 3.0: an updated microbial signal transduction database with an emphasis on chemosensory systems, Nucleic Acids Res. 48 (2020) D459–D464. https://doi.org/10.1093/nar/gkz988.

[6] R.P. Alexander, I.B. Zhulin, Evolutionary genomics reveals conserved structural determinants of signaling and adaptation in microbial chemoreceptors, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 2885–2890. https://doi.org/10.1073/pnas.0609359104.

[7] J. Lacal, C. García-Fontana, F. Muñoz-Martínez, J.-L. Ramos, T. Krell, Sensing of environmental signals: classification of chemoreceptors according to the size of their ligand binding regions, Environ. Microbiol. 12 (2010) 2873–2884. https://doi.org/10.1111/j.1462-2920.2010.02325.x.

[8] V. Anantharaman, L. Aravind, Cache - a signaling domain common to animal Ca(2+)-channel subunits and a class of prokaryotic chemotaxis receptors, Trends Biochem. Sci. 25 (2000) 535–537. https://doi.org/10.1016/s0968-0004(00)01672-8.

[9] A.A. Upadhyay, A.D. Fleetwood, O. Adebali, R.D. Finn, I.B. Zhulin, Cache Domains That are Homologous to, but Different from PAS Domains Comprise the Largest Superfamily of Extracellular Sensors in Prokaryotes, PLoS Comput. Biol. 12 (2016) e1004862. https://doi.org/10.1371/journal.pcbi.1004862.

[10] Á. Ortega, I.B. Zhulin, T. Krell, Sensory Repertoire of Bacterial Chemoreceptors, Microbiol. Mol. Biol. Rev. 81 (2017). https://doi.org/10.1128/MMBR.00033-17.

[11] A.F. Gasperotti, M.K. Herrera Seitz, R.S. Balmaceda, L.M. Prosa, K. Jung, C.A. Studdert, Direct binding of benzoate derivatives to two chemoreceptors with Cache sensor domains in *Halomonas titanicae* KHS3, Mol Microbiol. (2020). https://doi.org/10.1111/mmi.14630. Epub ahead of print. PMID: 33098326.

[12] L.E. Ulrich, I.B. Zhulin, Four-helix bundle: a ubiquitous sensory module in prokaryotic signal transduction, Bioinformatics. 21 Suppl 3 (2005) iii45-48. https://doi.org/10.1093/bioinformatics/bti1204.

[13] J.T. Henry, S. Crosson, Ligand-binding PAS domains in a genomic, cellular, and structural context, Annu. Rev. Microbiol. 65 (2011) 261–286. https://doi.org/10.1146/annurev-micro-121809-151631.

[14] J.S. Parkinson, G.L. Hazelbauer, J.J. Falke, Signaling and sensory adaptation in *Escherichia coli* chemoreceptors: 2015 update, Trends Microbiol. 23 (2015) 257–266. https://doi.org/10.1016/j.tim.2015.03.003.

[15] L.D. Miller, M.H. Russell, G. Alexandre, Diversity in bacterial chemotactic responses and niche adaptation, Adv. Appl. Microbiol. 66 (2009) 53–75. https://doi.org/10.1016/S0065-2164(08)00803-4.

[16] J. Lacal, F. Muñoz-Martínez, J.-A. Reyes-Darías, E. Duque, M. Matilla, A. Segura, J.-J.O. Calvo, C. Jímenez-Sánchez, T. Krell, J.L. Ramos, Bacterial chemotaxis towards aromatic hydrocarbons in *Pseudomonas*, Environ. Microbiol. 13 (2011) 1733–1744. https://doi.org/10.1111/j.1462-2920.2011.02493.x.

[17] S. Bencharit, M.J. Ward, Chemotactic responses to metals and anaerobic electron acceptors in *Shewanella oneidensis* MR-1, J. Bacteriol. 187 (2005) 5049–5053. https://doi.org/10.1128/JB.187.14.5049-5053.2005.

[18] C. Baraquet, L. Théraulaz, C. Iobbi-Nivol, V. Méjean, C. Jourlin-Castelli, Unexpected chemoreceptors mediate energy taxis towards electron acceptors in *Shewanella oneidensis*, Mol. Microbiol. 73 (2009) 278–290. https://doi.org/10.1111/j.1365-2958.2009.06770.x.

[19] J. Armitano, C. Baraquet, V. Michotey, V. Méjean, C. Jourlin-Castelli, The chemical-in-μwell: a high-throughput technique for identifying solutes eliciting a chemotactic response in motile bacteria, Res. Microbiol. 162 (2011) 934–938. https://doi.org/10.1016/j.resmic.2011.03.001.

[20] M.K.G. Ehrhardt, S.L. Warring, M.L. Gerth, Screening Chemoreceptor-Ligand Interactions by High-Throughput Thermal-Shift Assays, Methods Mol. Biol. 1729 (2018) 281–290. https://doi.org/10.1007/978-1-4939-7577-8_22.

[21] M. Fernández, Á. Ortega, M. Rico-Jiménez, D. Martín-Mora, A. Daddaoua, M.A. Matilla, T. Krell, High-Throughput Screening to Identify Chemoreceptor Ligands, Methods Mol. Biol. 1729 (2018) 291–301. https://doi.org/10.1007/978-1-4939-7577-8_23.

[22] A. Hida, T. Tajima, J. Kato, Two citrate chemoreceptors involved in chemotaxis to citrate and/or citrate-metal complexes in *Ralstonia pseudosolanacearum*, J. Biosci. Bioeng. 127 (2019) 169–175. https://doi.org/10.1016/j.jbiosc.2018.07.014.

[23] C. Bordi, C. Iobbi-Nivol, V. Méjean, J.-C. Patte, Effects of ISSo2 insertions in structural and regulatory genes of the trimethylamine oxide reductase of *Shewanella oneidensis*, J. Bacteriol. 185 (2003) 2042–2045. https://doi.org/10.1128/jb.185.6.2042-2045.2003.

[24] M. Herrero, V. de Lorenzo, K.N. Timmis, Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria., J Bacteriol. 172 (1990) 6557–6567. https://doi.org/10.1128/jb.172.11.6557-6567.1990.

[25] D.H. Figurski, D.R. Helinski, Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 1648–1652. https://doi.org/10.1073/pnas.76.4.1648.

[26] L.M. Guzman, D. Belin, M.J. Carson, J. Beckwith, Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter, J. Bacteriol. 177 (1995) 4121–4130. https://doi.org/ 10.1128/jb.177.14.4121-4130.1995.

[27] J. Armitano, V. Méjean, C. Jourlin-Castelli, Aerotaxis governs floating biofilm formation in *Shewanella oneidensis*, Environ. Microbiol. 15 (2013) 3108–3118. https://doi.org/10.1111/1462-2920.12158.

[28] R.K. Appleyard, Segregation of New Lysogenic Types during Growth of a Doubly Lysogenic Strain Derived from *Escherichia coli* K12, Genetics. 39 (1954) 440–452. https://doi.org/

[29] C. Gambari, A. Boyeldieu, J. Armitano, V. Méjean, C. Jourlin-Castelli, Control of pellicle biogenesis involves the diguanylate cyclases PdgA and PdgB, the c-di-GMP binding protein MxdA and the chemotaxis response regulator CheY3 in *Shewanella oneidensis*, Environ. Microbiol. 21 (2019) 81–97. https://doi.org/10.1111/1462-2920.14424.

[30] H.S. Yu, M. Alam, An agarose-in-plug bridge method to study chemotaxis in the Archaeon *Halobacterium salinarum*, FEMS Microbiol. Lett. 156 (1997) 265–269. https://doi.org/10.1111/j.1574-6968.1997.tb12738.x.

[31] G. Vardar, P. Barbieri, T.K. Wood, Chemotaxis of *Pseudomonas stutzeri* OX1 and *Burkholderia cepacia* G4 toward chlorinated ethenes, Appl. Microbiol. Biotechnol. 66 (2005) 696–701. https://doi.org/10.1007/s00253-004-1685-4.

[32] K.D. Collins, T.M. Andermann, J. Draper, L. Sanders, S.M. Williams, C. Araghi, K.M. Ottemann, The *Helicobacter pylori* CZB Cytoplasmic Chemoreceptor TlpD Forms an Autonomous Polar Chemotaxis Signaling Complex That Mediates a Tactic Response to Oxidative Stress, J. Bacteriol. 198 (2016) 1563–1575. https://doi.org/10.1128/JB.00071-16.

[33] V. Korolik, K.M. Ottemann, Two Spatial Chemotaxis Assays: The Nutrient-Depleted Chemotaxis Assay and the Agarose-Plug-Bridge Assay, Methods Mol. Biol. 1729 (2018) 23–31. https://doi.org/10.1007/978-1-4939-7577-8_3.

[34] C. Roggo, E.E. Clerc, N. Hadadi, N. Carraro, R. Stocker, J.R. van der Meer, Heterologous Expression of *Pseudomonas putida* Methyl-Accepting Chemotaxis Proteins Yields *Escherichia coli* Cells Chemotactic to Aromatic Compounds, Appl. Environ. Microbiol. 84 (2018). https://doi.org/10.1128/AEM.01362-18.

[35] Y. Kalinin, S. Neumann, V. Sourjik, M. Wu, Responses of *Escherichia coli* bacteria to two opposing chemoattractant gradients depend on the chemoreceptor ratio, J. Bacteriol. 192 (2010) 1796–1800. https://doi.org/10.1128/JB.01507-09.

[36] M.A. Machuca, A. Roujeinikova, Method for Efficient Refolding and Purification of Chemoreceptor Ligand Binding Domain, J Vis Exp. 130 (2017) 57092. https://doi.org/10.3791/57092.

[37] R.B. Kapust, D.S. Waugh, *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused, Protein Sci. 8 (1999) 1668–1674. https://doi.org/10.1110/ps.8.8.1668.

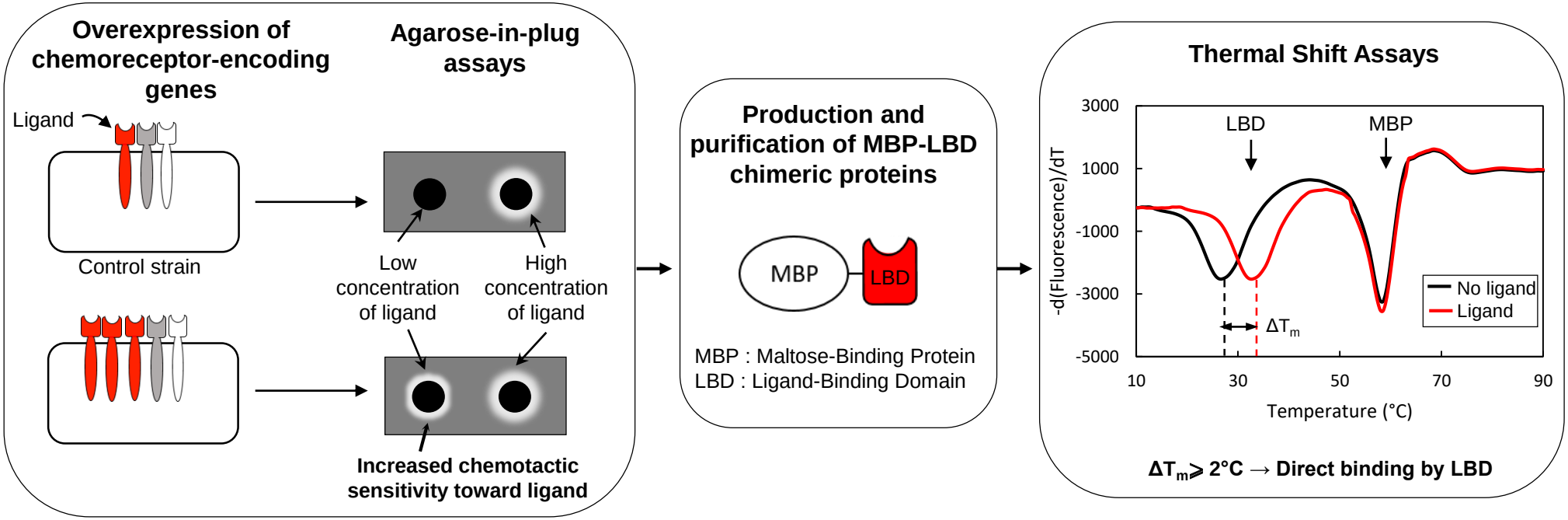
[38] S. Raran-Kurussi, D.S. Waugh, The ability to enhance the solubility of its fusion partners is an intrinsic property of maltose-binding protein but their folding is either spontaneous or chaperone-mediated, PLoS ONE. 7 (2012) e49589. https://doi.org/10.1371/journal.pone.0049589.

[39] F.H. Niesen, H. Berglund, M. Vedadi, The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability, Nat Protoc. 2 (2007) 2212–2221. https://doi.org/10.1038/nprot.2007.321.

[40] D. Martín-Mora, A. Ortega, J.A. Reyes-Darias, V. García, D. López-Farfán, M.A. Matilla, T. Krell, Identification of a Chemoreceptor in *Pseudomonas aeruginosa* That Specifically Mediates Chemotaxis Toward α-Ketoglutarate, Front Microbiol. 7 (2016) 1937. https://doi.org/10.3389/fmicb.2016.01937.

[41] J.L.O. McKellar, J.J. Minnell, M.L. Gerth, A high-throughput screen for ligand binding reveals the specificities of three amino acid chemoreceptors from *Pseudomonas syringae* pv. *actinidiae*, Mol Microbiol. 96 (2015) 694–707. https://doi.org/10.1111/mmi.12964.

[42] H. Baaziz, C. Gambari, A. Boyeldieu, A. Ali Chaouche, R. Alatou, V. Méjean, C. Jourlin-Castelli, M. Fons, ChrASO, the chromate efflux pump of *Shewanella oneidensis*, improves chromate survival and reduction, PLoS ONE. 12 (2017) e0188516. https://doi.org/10.1371/journal.pone.0188516.


Overexpression of chemoreceptor-encoding genes
Agarose-in-plug assays
Ligand
Control strain
Low concentration of ligand
High concentration of ligand
Increased chemotactic sensitivity toward ligand
Production and purification of MBP-LBD chimeric proteins
MBP
LBD
MBP : Maltose-Binding Protein
LBD : Ligand-Binding Domain
Thermal Shift Assays
-d(Fluorescence)/dT
3000
1000
-1000
-3000
-5000
10
30
50
70
90
Temperature (°C)
LBD
MBP
ΔTm
No ligand
Ligand
ΔTm ⩾ 2°C → Direct binding by LBD