

Identification and Characterization of Bacterial Chemoreceptors Using Quantitative Capillary and Gradient Plate Chemotaxis Assays

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[Abstract] Bacterial chemotaxis is a motility-based response that biases cell movement toward beneficial molecules, called attractants, and away from harmful molecules, also known as repellents. Since the species of the genus *Pseudomonas* are characterized by a metabolic versatility, these bacteria have developed chemotactic behaviors towards a wide range of different compounds. The specificity of a chemotactic response is determined by the chemoreceptor, which is at the beginning of the signaling cascade and which receives the signal input. The basic elements of a typical chemoreceptor are the periplasmic ligand binding domain (LBD), responsible for sensing environmental stimuli, and the cytosolic methyl-accepting (MA) domain, that interacts with other components of the cellular signaling cascade. *Escherichia coli* (*E. coli*), the traditional model in chemotaxis research, has 5 well-characterized chemoreceptors. However, genome sequence analyses have revealed that many other bacteria possess many more chemoreceptors, some of which with partially overlapping signal profiles. This high number of chemoreceptors complicates their study by the analysis of single chemoreceptor mutants. We have pursued an alternative strategy for chemoreceptor characterization which corresponds to the generation of chimeric receptors composed of the LBD of the chemoreceptor under investigation and the MA domain of an *E. coli* receptor (Tar). The chimera is then introduced into a chemoreceptor free mutant of *E. coli* and the chemotaxis of the resulting strain is entirely due to the action of this chimeric receptor. In this publication we describe the use of quantitative capillary and gradient plate assays to study *Pseudomonas* chemotaxis as well as *E. coli* strains harboring chimeric receptors.

Materials and Reagents

A. Materials

1. Sterilin™ Standard 90 mm Petri Dishes (Thermo Fisher Scientific, catalog number: 101/IRR)
2. Microtest plate 96-well, F (SARSTEDT AG & Co, catalog number: 82.1581.501)
3. 1.5 ml Eppendorf tubes

4. Square petri dishes (120 mm x 120 mm) with grid (Greiner Bio-One GmbH, catalog number: 688102)
5. Capillaries (Sigma-Aldrich, Drummond Microcaps®, catalog number: P1424)
6. Bulb for Pasteur pipette
7. Erlenmeyer flasks 100 ml

B. Strains

1. *P. aeruginosa* PAO1 (Stover *et al.*, 2000)
2. *E. coli* HD49 (Reyes-Darias *et al.*, 2015a), chemoreceptor free strain *E. coli* UU1250 (Ames *et al.*, 2002) harboring a plasmid encoding a chimeric receptor comprising the LBD of the PctC chemoreceptor of *P. aeruginosa* PAO1 (Taguchi *et al.*, 1997; Rico-Jimenez *et al.*, 2013) and the MA domain of the *E. coli* Tar receptor.

C. Reagents

1. HEPES sodium salt (Sigma-Aldrich, catalog number: H7006)
2. Potassium phosphate dibasic (HK₂PO₄) (Sigma-Aldrich, catalog number: P3786)
3. Sodium salicylate (Sigma-Aldrich, catalog number: S3007)
4. Chloramphenicol (Sigma-Aldrich, catalog number: C-0378)
5. Potassium phosphate monobasic (H₂KPO₄) (Sigma-Aldrich, catalog number: 60220)
6. Ammonium sulfate [(NH₄)₂SO₄] (Merck Millipore Corporation, catalog number: 1.01217)
7. Sodium citrate tribasic dihydrate (Sigma-Aldrich, catalog number: C7254)
8. Magnesium sulfate heptahydrate (MgSO₄·7H₂O) (Sigma-Aldrich, catalog number: 63140)
9. Thiamine hydrochloride (Thiamine HCl) (Sigma-Aldrich, catalog number: 47858)
10. Glycerol (Scharlab, S.L., catalog number: GL0027005P)
11. L-threonine (Sigma-Aldrich, Fluka, catalog number: 89179)
12. L-methionine (Sigma-Aldrich, catalog number: M9625)
13. L-leucine (Merck Millipore Corporation, catalog number: 5360)
Note: Currently, it is "Merck Millipore Corporation, catalog number: 105360".
14. L-histidine (Sigma-Aldrich, catalog number: 153688)
15. γ-Aminobutyric acid (GABA) (Sigma-Aldrich, catalog number: A2129)
16. Bacto-Agar (BD, Difco™, catalog number: 281230)
17. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
18. Tryptone Broth (TB) medium (see Recipes)
19. 0.9% NaCl solution (see Recipes)
20. LB medium (see Recipes)
21. 5x minimal A salts (see Recipes)
22. 5 mg/ml Aminoacid-mix (see Recipes)
23. Minimal A gradient plate medium (see Recipes)

Equipment

1. Spectrophotometer (Perkin Elmer, model: uv/vis lambda 20)
2. Incubators (30 °C and 37 °C) (Thermo Fisher Scientific, Heraeus, model: B6060 incubator)
3. Orbital shaker incubator SH maxi (ControItécnica Instruments)
4. Centrifuge Allegra X-22R (Beckman Coulter)
5. pH meter GLP22 (HACH LANGE SPAIN, Crison)
6. Bunsen burner
7. Two pairs of tweezers
8. Bulb dispenser (Drummond Scientific Company, catalog number: 1-000-9000)

Procedure

In the first part of this publication we will describe the quantitative capillary assay using as an example the chemotaxis towards γ -aminobutyrate (GABA). These experiments have shown that *P. aeruginosa* PAO1 shows GABA chemotaxis and that this response is mediated by the PctC chemoreceptor (Reyes-Darias *et al.*, 2015a). To characterize in more detail the PctC chemoreceptor, we have produced a chimeric receptor (Figure 1), which is reported in further detail (Reyes-Darias *et al.*, 2015a). There is now a significant body of information showing that different receptor chimera constructs are functional (Feng *et al.*, 1997; Krikos *et al.*, 1985; Kristich *et al.*, 2003; Repik *et al.*, 2000; Reyes-Darias *et al.*, 2015a; Reyes-Darias *et al.*, 2015b; Weerasuriya *et al.*, 1998).

In the second part of this publication we will describe the gradient plate chemotaxis assay on the example of the chemoreceptor free strain *E. coli* UU1250 into which a construct encoding the PctC-Tar chimera had been introduced. This approach is a convenient means to determine the chemoeffector profile by *in vivo* experimentation.

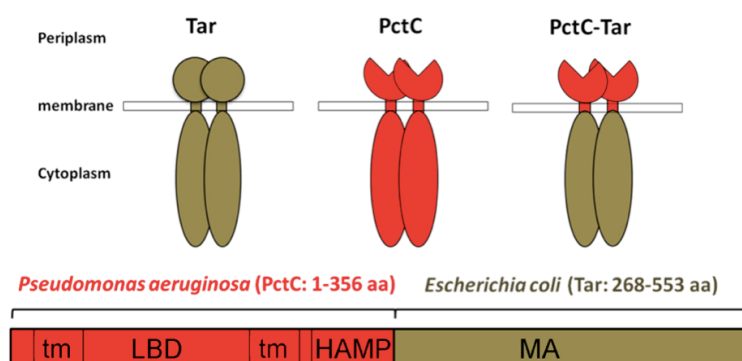


Figure 1. Construction of the PctC-Tar chimeric receptor. Tm: Transmembrane region; LBD: ligand binding domain; The HAMP linker domain (present in Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases) is an approximately

50-amino acid alpha-helical region; MA: Methylaccepting domain; Modified version of Figure taken from (Reyes-Darias *et al.*, 2015a).

Part I. Quantitative capillary chemotaxis assay

The principle of this assay consists of immersing chemoeffector filled capillaries into a bacterial suspension. In the case of chemotaxis, cells will preferentially swim into the capillary whereas in the case of chemorepulsion, fewer cells will swim into the capillary as compared to the buffer filled control capillary. Capillaries are then emptied and the number of colony forming units is determined. We detail here a modified version of the original capillary assay developed by Adler (1973).

1. *P. aeruginosa* PAO1 is grown overnight (12-18 h) in LB medium (pH=7.4) on a rotator shaker at 200 rpm and 37 °C.
2. Subsequently 0.1-0.2 ml overnight culture is transferred to 20 ml of fresh LB medium (pH=7.4) in 100 ml Erlenmeyer flasks. The initial OD₆₀₀ should be between 0.05-0.07.
3. The culture is grown to early stationary phase (OD₆₀₀ 0.25-0.35) at 37 °C and 200 rpm.
4. The cells then have to be changed to an appropriate medium for mobility and chemotaxis. Wash twice 4 ml of the culture with four ml of chemotaxis buffer (HEPES, pH=7.0) by centrifuging at 1,667 x g (4 °C) for 5 min, followed by resuspension of cell pellet to a density of OD₆₀₀= 0.04-0.05 in 4-8 ml of chemotaxis buffer. Harsh cell treatment may result in the loss of the flagella.
5. Subsequently, 230 µl aliquots of bacterial suspension are placed into the wells of a 96-well plate.
6. Capillaries are heat-sealed at one end over the flame of a burner (Figure 2A). The open end is then inserted into the chemoattractant solution (Figure 2B), of which the pH had been adjusted to that measured for the bacterial suspension (pH=7.0). As negative controls, capillaries are filled with chemotaxis buffer and for positive controls, capillaries are filled with a known, strong chemoattractant such, in the case of *Pseudomonas* strains, with 0.1 % of casamino acids. The closed end of the capillary is inserted into the rubber adaptor, which is then placed onto the wells in a way that the open end of the capillary is immersed into the bacterial suspension (Figure 2C).
7. After incubation for 30 min, the capillaries are removed from the plate and the section of the capillary that was in contact with bacteria is rinsed with water. The sealed end of the capillary is broken and its contents emptied into a microfuge tube containing 1 ml of 0.9% (w/v) NaCl solution (Figure 2D) using a bulb dispenser.

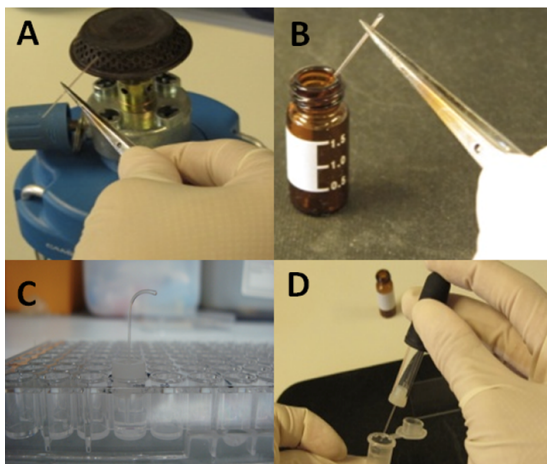


Figure 2. Experimental setup of capillary assays. A. One end of the capillary is sealed on open fire. B. Sealed and warmed capillary is inserted into the chemoattractant solution for filling. C. The rubber adaptor with the capillary is placed onto the well, which submerges the open end of capillary into the bacterial suspension that had been placed previously into the well. D. The capillary content is emptied into an Eppendorf tube containing 0.9% (w/v) NaCl.

8. After a short centrifugation (5-10 sec), serial 10-fold dilutions of the resulting cell suspension are prepared in 0.9% (w/v) NaCl. For cell counting, a modified version of the protocol described in (Hoben and Somasegaran, 1982) is used. Agar plates containing LB medium (pH=7.4) supplemented with 17.5 $\mu\text{g/ml}$ chloramphenicol are divided into six sectors, and three individual 20 μl aliquots are placed onto a plate sector as shown in Figure 3.
9. Plates are incubated at 37 $^{\circ}\text{C}$ for 20-24 h prior to cell counting.

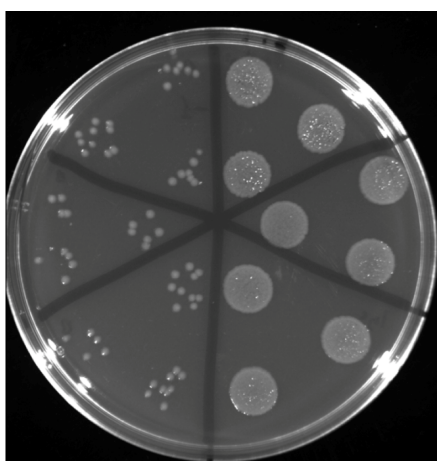


Figure 3. LB agar plates containing chloramphenicol used for cell counting in the quantitative capillary chemotaxis assay. *P. aeruginosa* PAO1 colonies from the buffer control (left) and from chemotaxis towards 1 mM GABA (right). Shown are 10-fold dilutions

and further dilutions are necessary to precisely quantify colonies of the GABA chemotaxis experiment.

Part II. Gradient plate assays

In this assay aliquots of chemoeffectors are deposited on an agar plate. After incubation permitting chemoeffector gradient formation, aliquots of the bacterial suspension are placed at a defined distance from the deposited chemoeffector. After approx. 1 day, plates are inspected. In the case of chemoattraction, the bacterial halo is acentric towards the chemoeffector, in the case of chemorepulsion the halo is acentric away from the chemoeffector.

1. Square petri dishes (120 mm x 120 mm) with vents are filled with 80 ml of Minimal A gradient plate medium (pH=7) (see Note 1). Plates are cooled at room temperature for at least 3 h. Care must be taken when moving the plates since the agar is semi-solid and can be damaged easily.
2. Along the vertical central line of the plate, 10 μ l aliquots of a concentrated chemoeffector solution (10 mM GABA in the case shown, see Note 2), dissolved in sterile water, are placed at regular distances (Figure 4). Plates are incubated for 12-16 h at 4 °C for gradient formation.
3. Bacteria, in the case shown *E. coli* HD49, are grown overnight in TB medium supplemented with 17.5 μ g/ml chloramphenicol at 30 °C.
4. 1 ml of the cell culture is then washed twice with 1 ml of 0.9 % (w/v) NaCl by consecutive centrifugation at 1,667 x g at 4 °C for 5 min and resuspension. The cell suspension is then diluted with 0.9% (w/v) NaCl to an OD₆₀₀ of 0.4-0.6.
5. Initially, 2 microliter aliquots of bacterial suspension are placed horizontally to each of the chemoattractant spots but with varying distances of 0.5-3.5 cm to the chemoeffector deposit (Figure 4). Plates are incubated at 30 °C for 16-30 h. The distance at which chemotaxis is seen best is then used for further analyses. Typically, cells can be deposited on either side of the attractant, which is a way to generate duplicate measurements or to assess taxis of different strains (wt and mutant strain) towards the same chemoeffector deposit.
6. From these plates the magnitude of chemotaxis can be determined in a semi-quantitative manner by calculating the response index as described by (Pham and Parkinson, 2011). Briefly, the distances from the site of inoculation to the colony edges closest to (D1) and furthest from (D2) the chemoattractant source are determined to calculate the response index (RI) using $RI = D1/(D1 + D2)$. RIs superior to 0.52 indicate chemotaxis, whereas RIs inferior to 0.48 represent chemorepulsion. RI values between 0.48 and 0.52 indicate neutral behavior.

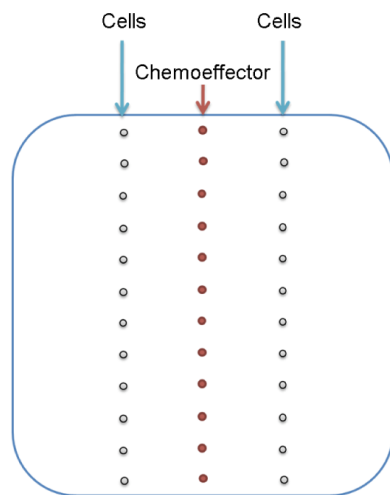


Figure 4. The experimental set-up of the gradient plate assay

Representative data

Figure 5 shows results from quantitative capillary assays of *P. aeruginosa* PAO1, its mutant deficient in the PctC chemoreceptor and the mutant complemented with a plasmid harboring the *mcpG* gene towards GABA. Data were corrected with the number of cells that swam into buffer containing capillaries and are expressed as bacterial cells that migrate into the GABA containing capillary.

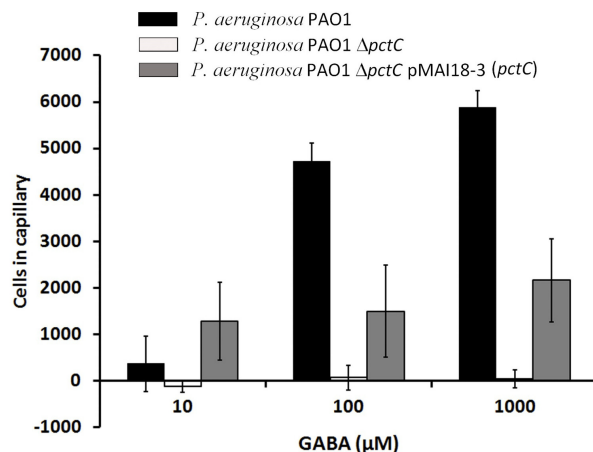


Figure 5. Quantitative capillary chemotaxis assays of wild type, mutant and complemented mutant strains of *P. aeruginosa* PAO1 to GABA. This figure was taken from Rico-Jimenez *et al.* (2013).

Figure 6 shows gradient plate assays towards GABA of *E. coli* strains containing either the Tar receptor or the PctC-Tar chimera as a sole chemoreceptor. In the case of Tar, the

bacterial halo is circular and consequently an RI of 0.50 was determined indicating the absence of chemotaxis. In the PctC-Tar experiment, the halo is acentric towards deposited GABA and the derived RI of 0.88 indicates strong chemotaxis.

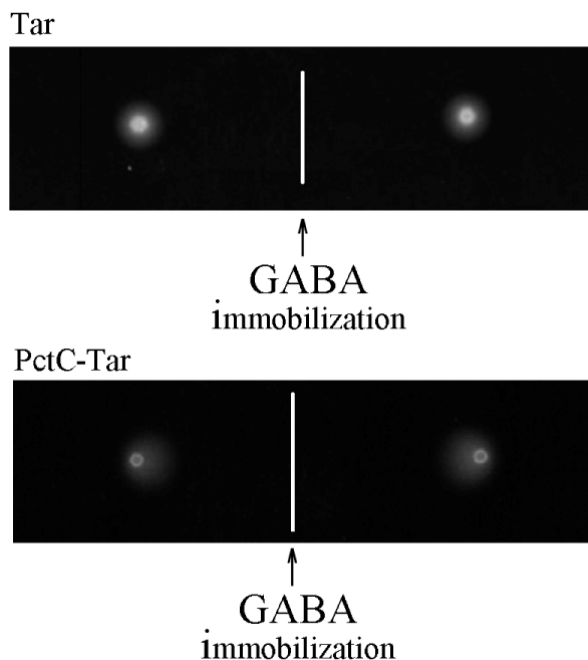


Figure 6. Gradient plate assay of *E. coli* expressing either Tar (top) or PctC-Tar (bottom) as the only chemoreceptor. Shown are duplicate samples. The response indices were 0.5 (\pm 0.05) for Tar and 0.88 (\pm 0.02) for PctC-Tar. Modified version of a figure is taken from Reyes-Darias *et al.* (2015a).

Notes

1. The plate contained 1 μ M sodium salicylate to induce expression of the chimeric receptors and 17.5 μ g/ml chloramphenicol to select for plasmid maintenance.
2. The optimal attractant concentration in the gradient plate assay varies. Initial experiments with different concentrations for chemoeffector deposit may be carried out (1 to 100 mM). Frequently, good responses are observed with 10 mM solutions and a distance of 2.5 cm between bacteria and the chemoeffector.
3. Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 $^{\circ}$ C) and analytical grade reagents.

Recipes

Note: Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25 $^{\circ}$ C) and analytical grade reagents.

1. Tryptone Broth (TB) medium
 - 10 g tryptone
 - 5 g NaCl
 - Add dH₂O to 1,000 ml
 - Autoclaved and stored at room temperature
2. 0.9% NaCl solution
 - 9 g of NaCl
 - Add dH₂O to 1,000 ml
 - Autoclaved and stored at room temperature
3. Luria-Bertani (LB) medium
 - 5 g yeast extract
 - 10 g tryptone broth medium
 - 5 g NaCl
 - Add dH₂O to 1,000 ml
 - Adjust pH to 7.4
 - Autoclaved and stored at room temperature
4. 5x minimal A salts
 - 26.25 g K₂HPO₄
 - 11.25 g KH₂PO₄
 - 2.5 g (NH₄)₂SO₄
 - 1.25 g Na citrate
 - Add dH₂O to 500 ml
 - Autoclaved and stored at room temperature
5. 5 mg/ml Aminoacid-mix
 - 250 mg of L-threonine
 - 250 mg of L-histidine
 - 250 mg L-methionine
 - 250 mg L-leucine
 - dH₂O to 50 ml
 - Filtered and stored at 4 °C
6. Minimal A gradient plate medium
 - 1x minimal A salts
 - 0.2% (v/v) glycerol
 - 1 mM MgSO₄·7H₂O
 - 0.04 mg/ml aminoacid-mix
 - 0.1 mg/ml thiamine
 - 0.25% (w/v) agar
 - dH₂O to 100 ml
 - Adjust pH to 7
 - Gradient plates are allowed to solidify between 2-4 h at room temperature

Acknowledgments

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