Chemotaxis by *Pseudomonas putida* (ATCC 17453) Towards Camphor Involves Cytochrome $P450_{cam}$ (CYP101A1)

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Abstract

The camphor-degrading microorganism, *Pseudomonas putida* strain ATCC 17453, is an aerobic, gram-negative soil bacterium that uses camphor as its sole carbon and energy source. The genes responsible for the catabolic degradation of camphor are encoded on the extra-chromosomal CAM plasmid. A monooxygenase, cytochrome $P450_{cam}$, mediates hydroxylation of camphor to 5-*exo*-hydroxycamphor as the first and committed step in the camphor degradation pathway, requiring a dioxygen molecule (O_2) from air. Under low O_2 levels, $P450_{cam}$ catalyzes the production of borneol *via* an unusual reduction reaction. We have previously shown that borneol downregulates the expression of $P450_{cam}$. To understand the function of $P450_{cam}$ and the consequences of down-regulation by borneol under low O_2 conditions, we have studied chemotaxis of camphor induced and non-induced *P. putida* strain ATCC 17453. We have tested camphor, borneol, oxidized camphor metabolites and known bacterial attractants (D)-glucose, (D) - and (L)-glutamic acid for their elicitation chemotactic behavior. In addition, we have used 1-phenylimidazole, a $P450_{cam}$ inhibitor, to investigate if $P450_{cam}$ plays a role in the chemotactic ability of *P. putida* in the presence of camphor. We found that camphor, a chemoattractant, became toxic and chemorepellent when $P450_{cam}$ was inhibited. We have also evaluated the effect of borneol on chemotaxis and found that the bacteria chemotaxed away from camphor in the presence of borneol. This is the first report of the chemotactic behaviour of *P. putida* ATCC 17453 and the essential role of $P450_{cam}$ in this process.

Keywords

Bacterial chemotaxis, CYP101A1, *Pseudomonas putida*, Aerobic soil bacteria, Camphor, Borneol.

1. Introduction

Chemotaxis is the ability of motile bacteria to respond to changes in the concentration of chemical compounds. Motile bacteria are attracted towards chemical substances that are beneficial to them if they can be used as a carbon or energy source. Conversely, they show chemorepulsion and move away from chemicals that are either not advantageous or toxic to them.[1, 2] However, it is not always necessary that a compound be toxic for bacteria to chemotax away from it.[3] The *Pseudomonads* are known for their wide variety of substrate tolerance, due to the specific catabolic plasmids that enable them to grow on these substrates.[4-6] Ability to chemotax towards these specialized substrates provides them with an ecological advantage, namely, to minimize competition for these unique carbon and energy sources.[7] Soil bacteria, such as *Pseudomonas* species, show chemotactic mobility towards or away from various natural and unnatural substrates.[4, 8-18] There are several reports on the chemotactic ability of some of the *Pseudomonas* species towards specialized carbon sources.[4-6, 12-19] *Pseudomonas putida* ATCC 17453 (*Strain 1*) is a rod-shaped, gram-negative, soil bacterium that utilizes (1*R*) (+)-camphor **1** (**Fig. 1a**) as a carbon and energy source.[20, 21] For a wild soil bacterium like *Strain 1*, the ability to chemotax towards its carbon source, camphor (probably released by plants through their roots)[22] will pose an ecological advantage. ATCC 17453 exploits camphor as a carbon source by making use of a plasmid (CAM plasmid)[23], which encodes genes for the degradation of camphor. Cytochrome $P450_{cam}$ (CYP101A1) is well known for the regio- and stereo-selective hydroxylation of camphor at the 5^{th} position to give 5-*exo*-hydroxycamphor **2** using electrons from NADH, transferred to the P450 by the redox partners (putidaredoxin, PdX and putidaredoxin reductase, PdR).[24-26] CamD, a dehydrogenase in the camphor degradation pathway, was shown to catalyze the desaturation of 5-*exo*-hydroxycamphor to give 5-ketocamphor.[27] However, cytochrome $P450_{cam}$ by itself can perform a second round of oxidation to give 5-ketocamphor, depending on the level of O_2 present.[28-30] Expression of the camphor hydroxylase system ($P450_{cam}$, PdX, PdR, and CamD) is induced by (+/-)-camphor and other monoterpenes, such as 2-*endo*-hydroxybornane, 3-bornanone, 5-bornanone, 5-ketocamphor, and (+/-)-isoborneol.[31] Other monooxygenases encoded on the CAM plasmid catalyse successive Baeyer-Villiger oxidations to give an unstable lactone **4** that, upon spontaneous ring cleavage, forms 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid **5**. An acetyl-

CoA synthetase produces the corresponding acetyl-CoA analogue of the acid **6**, which undergoes Baeyer-Villiger oxidation to produce 3,4,4-trimethyl-Δ^3-pimelyl-CoA **7**. Ring cleavage of the lactone leads to the formation of isobutyryl-CoA and acetyl-CoA. These metabolic intermediates enter central metabolism as carbon and energy sources (**Fig 1**).[20, 32-35] Full camphor oxidation to central metabolites requires four molecules of O_2 per camphor molecule (including $P450_{cam}$ catalyzed 5-ketocamphor formation). Additional O_2 is required for the aerobic metabolism of the CoA esters formed from camphor. Therefore, camphor catabolism is an oxygen demanding process. We have observed that under low O_2 levels, $P450_{cam}$ catalyzes an abnormal reduction reaction on camphor to give borneol **8** (**Fig. 1b**). Furthermore, we noticed that borneol downregulates the expression of the $P450_{cam}$ hydroxylase system ($P450_{cam}$, PdR, PdX).[29, 36] Therefore, we have suggested that borneol could act as a signal that there is insufficient O_2 available to digest camphor fully (**Fig. 1**).[36]**Fig 1**

The layer of soil that contains plant roots (the rhizosphere) is reported to be a reservoir of volatile organic compounds (*e.g.*, camphor) released by plants through their roots.[22, 37-40] Soil bacteria like *P. putida* ATCC 17453 exploit such volatiles as specialized carbon sources.[41, 42] We hypothesize that these bacteria should chemotax towards camphor and selected metabolites released into media, such as 5-*exo*-hydroxycamphor **2** and 5-ketocamphor **3**. Furthermore, we hypothesize that (+)-borneol **8** should have a different effect from (+)-camphor **1**, as it appears to be a signal that there is insufficient O_2 to fully metabolize camphor.[29, 36] Here we report the chemotactic responses of *P. putida* ATCC 17453 towards (+)-camphor **1**, its metabolites (5-*exo*-hydroxycamphor **2** and 5-ketocamphor **3**),(+)-borneol **8**, L-glutamic acid **9** and D-glutamic acid **10**. This is the first report of chemotactic behaviour of *P. putida* strain ATCC 17453. In addition, we have investigated the role of $P450_{cam}$ in the chemotactic response, in experiments with a $P450_{cam}$ inhibitor (1-phenylimidazole **11**), the down-regulator (+)-borneol **8** and *P. putida* strains that do not contain the CAM plasmid and, therefore, lack cytochrome $P450_{cam}$.

2. Materials and methods

2.1 Chemicals and general methods

(+)-Borneol, (1*R*)-(+)-camphor, 1-phenylimidazole, (D)-glutamic acid, (L)-glutamic acid, (D)-glucose, agarose, and DMSO (molecular biology grade) were purchased from Sigma-Aldrich Canada (Oakville, Ontario). 5-*exo*-hydroxycamphor with other inseparable polar metabolites (which has 5-*exo*-hydroxycamphor as major component, and we call the "polar metabolite mix") and 5-ketocamphor were purified from the lysates of camphor-metabolizing *Pseudomonas putida* ATCC 17453 (*Strain 1*) as described earlier.[29, 43] Briefly, camphor-induced cultures of *P. putida* were centrifuged (7,000×g, 10 min) and the supernatant was extracted thrice with dichloromethane. Organic layers were combined, concentrated in vacuum and the crude product obtained was purified by silica gel column chromatography with hexane/ethyl acetate gradients. NMR data of the purified compounds have been deposited in the supplementary information (**Figs. S2** & **S3**).

NMR spectra were obtained on Bruker DRX 400 or 500 MHz instruments in $CDCl_3$ as solvent. Chemical shifts are reported as parts per million (ppm) from the 0 ppm (tetramethylsilane) reference. Gas chromatography-mass spectrometry (GC-MS) was done on a Varian CP-3800 GC interfaced with a Varian Saturn 2000 ion trap MS. The GC was fitted with a 30 m long fused silica capillary column SPB-5 (Supelco, Bellefonte, PA, USA) of 0.25 mm inner diameter and containing a film of 0.25 μm thickness. The carrier gas was helium, and the ionization was achieved through electron impact (EI). The GC was programmed as follows: 45 ^{o}C for 0.5 min, 7 ^{o}C/min to 180 ^{o}C (hold for 1 min), 20 ^{o}C/min to 260 ^{o}C (hold for 5 min). The split/splitless injector was programmed at 260^{o}C and used in splitless mode. The ionization was programmed as EI automatic (ionization current 30 μA), and ions were collected in SIS (specific ion storage) mode between m/z = 90 – m/z = 170 in the retention time window in which camphor, borneol, ketocamphor and 5-*exo* hydroxycamphor eluted (9.0 – 19.0 min). The instrument's response was calibrated using commercial (camphor, borneol) or synthesized (ketocamphor, 5-*exo* hydroxycamphor).[29]

Analysis of enzyme kinetics, dose responses and analysis of variance (ANOVA) were done using GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, CA, USA).

2.2 Microorganisms, growth conditions and media preparations

Three different *Pseudomonas putida* strains used, ATCC 17453 (*Strain 1*), ATCC 17484 (*Strain 2*), and ATCC 33015 (*Strain 3*), were purchased from Cedarlane (Burlington, Ontario). ATCC 17484 is a naphthalene-degrading strain.[5, 44] ATCC 33015 is a toluene and xylene-degrading strain with the TOL plasmid.[45-47] *Strains 2 & 3* do not contain the CAM plasmid and are not known to metabolize camphor. These strains served as a negative control. *Strains 1 & 2* were grown/propagated in nutrient broth (NB) media/plates. *Strain 3* was grown on benzoate medium.

2.3 Chemical-in-plug assays

Chemical-in-plug assays were performed using hard agar plugs containing 2% agar in chemotaxis buffer, CB buffer (10 mM potassium phosphate, 0.1 mM EDTA, pH 7.4), with the selected chemo effectors: (+)-camphor **1**, (+)-borneol **8**, camphor metabolites, (D)-glucose, (L)- or (D)-glutamic acid (**9** or **10**), and 1-phenylimidazole **11** (a known P450 inhibitor). Chemotaxis assays were performed with different *P. putida* strains: ATCC 17453 (*Strain 1*), ATCC 17484 (*Strain 2*), and ATCC 33015 (*Strain 3*). The cultures were grown until the OD_{600} reached 0.6 - 0.8. The bacterial cultures were harvested by low-speed centrifugation at 4,000 × g for 3 min in a Beckmann Avanti centrifuge (using a JLA 8.1000 rotor). The cell pellet was washed and re-suspended in the CB buffer. Sterile 0.35% agar solution in CB buffer was used to dissolve the harvested motile cells of *P. putida* strains (1, 2, or 3) to a final concentration of ≈ 4 × 10^9CFU/mL. The motile bacterial suspension was poured over the circular sections of hard agar plugs in a Petri-dish and allowed to solidify at room temperature, followed by incubation at 27 °C (for *Strains 1* and *2*) or at 30 °C (for *Strain 3*). The chemotactic response in the form of a ring around the plug was noticed in 4 – 8 h of incubation. The plates were stored at 4 °C until the images were captured and the response was measured. All the experiments were performed in triplicate at different concentrations of the chemoeffectors (10 to 150 mM). A negative control with 0 mM chemoeffector and positive controls with (D)-glucose and (D) - & (L)-glutamic acid were also prepared. The chemotactic response (attraction or repulsion) was measured and quantified using the dose-response curve to get EC_{50} values. A similar experimental series was

repeated with the induced cultures of ATCC 17453. When the bacterial growth reached 0.5 at 600 nm, the cultures were treated with 0.01 % (w/v) of (+)-camphor to induce the expression of the $P450_{cam}$ hydroxylase system.

Several in-plug assays were also done with the strains of *P. putida* (ATCC 17484 and ATCC 33015) that do not contain the CAM plasmid and are known not to metabolize camphor.

2.4 Capillary chemotaxis assay

The capillary assays were performed in a 96-well plate using a disposable 1 mL plastic syringe to hold the chemo-effector solution (in CB buffer with 0.05% glycerol). Wells in the 96-well plate contained the motile bacterial cells in chemotaxis buffer. *P. putida Strain 1* was grown until mid-logarithmic growth phase (OD_{600} of 0.6 – 0.8) in 50 mL of NB media at 27 °C, 150 rpm. The cells were harvested by centrifuging at 4000 × g for 3 min in a Beckmann Avanti centrifuge (using a JA 25.50 rotor). The cell pellet was washed twice with chemotaxis buffer (pH 7.0) as before. The washed cells were resuspended in chemotaxis buffer to an OD_{600} of 0.08, to make a suspension of motile bacterial cells, and this was transferred into the wells of the 96-well plate (chemotaxis chamber). The chemoeffector solution (100 µL) was taken up into the needle of a disposable 1 mL plastic syringe and inserted into the chemotaxis chamber containing the motile cell suspension. After 30 – 60 min of exposure, the exterior of the syringe was washed with the chemotaxis buffer. The contents of the syringe were carefully dispensed into a 1.5 mL sterile Eppendorf tube and diluted up to 200 µL using the chemotaxis buffer. The chemotactic response was quantified by plating 50 µL of the diluted capillary contents onto 1.5 % NB-agar plates. The plates were incubated overnight at 27 °C. On the next day, colonies were counted and the colony forming units (CFU) per mL of the culture used were calculated. The experiment was performed at different concentrations of chemoeffector in five replicates, to quantify the chemotactic response.

To check for camphor diffusion out of the needle, into the well, we performed a diffusion assay. Chemotaxis buffer with 1 mM camphor (200 µL) was placed in the needle and clean chemotaxis buffer (200 µL) was placed in the wells. After 60 min at (room temperature), the contents of the well were taken into a glass vial and extracted twice with chloroform (containing 7.2 µM 1-

indanone internal standard). The organic layers were pooled, dried over anhydrous magnesium sulfate and analyzed by GC-MS.

To check for borneol or 1-phenylimidazole diffusion from the well into the needle, we set up chemotaxis buffer with 1 mM 1-phenylimidazole or 1 mM borneol in the well (200 μL) and placed clean chemotaxis buffer in the needle (200 μL). After 60 min at (room temperature) the contents of the needle were dispensed into a glass vial, extracted and analyzed by GC-MS as described above. Both of these diffusion experiments were done in four replicates.

2.5 Survival dose response assays to determine the IC_{50} of (+)-camphor, (+)-borneol, and 1-phenylimidazole with P. putida *strains ATCC 17453, 17484, and 33015*

Overnight grown cultures of the *Strains* (*1, 2,* or *3*) were inoculated into the appropriate growth medium (NB or sodium benzoate) and incubated at 27 °C (for *Strains 1* and *2*) or at 30 °C (for *Strain 3*), 250 rpm until an OD_{600} of 0.1 is reached. Cultures were diluted 1:1000 for the IC_{50} determination. To the diluted culture samples varying concentrations (0 mM to 100 mM) of either (+)-camphor, (+)-borneol, or 1-phenylimidazole were added. We used DMSO (the solvent used for making the stock solutions of the compounds tested) only (vehicle control) with no substrate as the negative control. Assays were performed in triplicate for each concentration of each tested compound. Test cultures were incubated at 27 °C or 30 °C, 250 rpm for an hour after adding the compound, after which 10 μL of the treated cultures were plated on 1% NB-agar plates and incubated overnight at 27 °C or 30 °C. On the next day, the number of colonies was counted. The cell density was determined by calculating the number of CFU/mL of the original culture used for the assay, using the appropriate dilution factor. The IC_{50} values for (+)-camphor, (+)-borneol, and 1-phenylimidazole on all the three strains (*1, 2,* & *3*) of *P. putida* were calculated by plotting a graph of cell density *versus* log of the respective substrate concentrations. To assess camphor toxicity in the $P450_{cam}$-inhibited ATCC 17453 strain, the toxicity assay was also performed at various concentrations of camphor in the presence of 20 mM 1-phenylimidazole.

2.6 1-Phenylimidazole as a carbon source for ATCC 17453

To determine whether the $P450_{cam}$ inhibitor, 1-phenylimidazole, can serve as a carbon source for *P. putida* ATCC 17453, an overnight grown culture of ATCC 17453 was harvested by centrifuging at 7,000 × rpm in a Beckmann Avanti centrifuge (using a JA 25.50 rotor). The cell pellet was resuspended in M9 minimal media to an OD_{600} of 0.1. The 1-phenylimidazole (20 mM in DMSO) was added and the culture continued incubating at 27 °C, and 250 rpm. At regular intervals (0 to 96 h), the cell culture was sampled and diluted (1000 ×) in M9 minimal media. Diluted samples (10 µL) were plated on 1% NB-agar plates (containing 10 g of agar per liter of Difco NB media) and incubated overnight at 27 °C. The next day, the CFU/mL of the culture was determined. The experiment was performed in triplicate with 40% (D)-glucose as the positive control.

2.7 Growth and isolation of crude lysate of ATCC 17453 and enzyme kinetics

P. putida (ATCC 17453) was grown in NB broth until the OD_{600} reached 0.8 – 0.9. The cultures were then induced with 2 mM (+)-camphor for the expression of Cam A (PdR), Cam B (PdX), and Cam C ($P450_{cam}$). Incubation continued for an additional 12 h at 27 °C, and 250 rpm. The cells were harvested and resuspended in lysis buffer (50 mM potassium phosphate buffer, 200 mM KCl (pH 7.4)) with 1 mM (+)-camphor, DTT (1 mM), Na_2S and protease inhibitors (PMSF – 40 mg/L, AEBSF – 1 mg/L, leupeptin – 0.1 mg/L, and aprotinin – 0.1 mg/L). Lysozyme (100 mg/L) was added to this cell suspension, which was lysed by sonication (Branson Ultrasonics Sonifier at 50% duty cycle for 10 min). During sonication, the suspension was kept on a bed of ice. Crude protein lysate was harvested and then dialyzed against the lysis buffer at 4 °C for 20 h, refreshing the buffer once. Protein concentrations ($P450_{cam}$, PdR, and PdX) were measured spectroscopically by using the respective extinction coefficients and absorbances at A_{392} ($P450_{cam}$), A_{325} (PdX), and A_{454} (PdR). Additionally, the Fe-CO difference spectrum of the $P450_{cam}$ in the lysate was recorded. For enzyme kinetics, the dialyzed protein solution was stripped off camphor as follows. Crude protein solution in camphor containing lysis buffer (3 mL, 18 µM) was diluted to 15 mL in camphor-free stripping buffer (50 mM Tris/HCl, 50 mM KCl (pH 7.4)) with DTT, Na_2S and protease inhibitors as listed above and then centrifuged (at 7,000 × g in a Beckmann Avanti centrifuge with rotor JA 25.50) to a final volume of 3 mL (≈18 µM). This process of dilution and re-concentration was performed four times, using the lysis

buffer (without camphor) for the last batch of buffer exchange. Reaction mixtures for the enzyme kinetics experiment contained 1.8 μM $P450_{cam}$ in the lysis buffer (see above) with varying (*1R*)-(+)-camphor concentration (0 to 1 mM) in the presence (1mM) or absence (0 mM) of 1-phenylimidazole (a P450 inhibitor) to establish the nature of inhibition. For the inhibition assay, 1-phenylimidazole was taken in varying concentrations (0 to 1 mM) in the presence of 1 mM (*1R*)-(+)-camphor. The reaction was initiated by adding 250 μM NADH and continued at room temperature for 20 min. After 20 min, the reaction mixture was extracted in chloroform (with 1-indanone, 7.2 μM, as the internal standard) for GC-MS quantification.

3. Results

3.1 In-plug chemotaxis assays of P. putida *strains (ATCC 17453, 17484, & 33015)*

The chemotactic responses of three wild, aerobic,*P. putida*soil bacterial strains *1*, *2*, &*3* (ATCC 17453, 17484, & 33015) towards (+)-camphor, (+)-borneol, 5-*exo*-hydroxycamphor ("polar metabolite mix"), 5-ketocamphor, 1-phenylimidazole and known bacterial chemoeffectors (D)-glucose, (D)- and (L)-glutamic acid were measured by in-plug assays. Chemoattraction manifested itself in the form of a circular ring of cells around the plug, whereas chemorepulsion was visualized as a clearing zone around the chemoeffector plug. Chemotaxis assays were performed with both, $P450_{cam}$-induced and non-induced *Strain 1* to understand the effect of $P450_{cam}$ on the chemotactic behaviour of *Strain 1*.The induced cells were thoroughly washed, to remove camphor and metabolites, before using in the assay. In general,*Strain 1*exhibited chemoattraction towards (D)-glucose, (+)-camphor, and 5-ketocamphor. This strain showed repulsion from (+)-borneol, 1-phenylimidazole and mixed responses towards 5-*exo*-hydroxycamphor ("polar metabolite mix") and glutamic acid enantiomers (**Table 1** & **Fig S4**). Importantly, *Strain 1* showed stronger chemorepulsion from borneol with the $P450_{cam}$–non-induced culture compared to $P450_{cam}$-induced *Strain 1*. Also, $P450_{cam}$-induced *Strain 1* exhibited relatively stronger chemoattraction towards camphor than the non-induced culture. There is not much difference between the responses towards glucose and glutamic acids by the $P450_{cam}$-induced and non-induced *Strain 1*(**Table 1** & **Fig S4**). The chemotactic responses were dose-dependent (**Fig S5** & **S6**). With camphor, *P. putida* ATCC 17453, showed a marble-like pattern

on the plates due to it acting as a carbon source that would increase the biomass and simultaneously create local anoxic conditions. The latter could trigger the formation of borneol and a downregulation in the CAM pathway, leading to the formation of randomly localized chemorepulsive regions, resulting in a marble-like chemoresponse pattern for camphor. To determine whether the marble-like pattern was due to the low solubility of camphor in the agarose plug, detergent (Tween-20) was used to help solubilize camphor. Chemical-in-plug experiments with 3% Tween-20 containing hard agarose plugs with varying concentrations of camphor displayed uniform chemoattraction rings after 8 hours of incubation. However, plugs without camphor but with Tween-20 also showed chemoattraction, which indicated that *P. putida* was attracted towards Tween-20. We left the plates in the incubator for several days and calculated the net chemoattraction towards camphor (subtracting the response to Tween-20 alone). However, all other responses in the in-plug assays were observed within 4 - 8 h of exposure of the bacteria to a chemical signal. Therefore, we decided to do a capillary assay to obtain dose responses for camphor chemotaxis.

Chemical-in-plug assays with the strains, ATCC 17484 (naphthalene degrading catabolic pathway) and ATCC 33015 (TOL plasmid, xylene metabolizing), not containing $P450_{cam}$ showed chemorepulsive responses, away from camphor and borneol (**Table 1 and Fig S7**). This behavior might be due to these strains not having the catalytic machinery (unlike *P. putida*, ATCC 17453) to metabolize camphor.

Table 1

3.2 Role of $P450_{cam}$ in the chemotaxis of P. putida (ATCC 17453) towards camphor

To investigate the role of $P450_{cam}$ in the chemotactic ability of *P. putida* ATCC 17453 towards camphor, 1-phenylimidazole (a known P450 inhibitor) was used to chemically inactivate $P450_{cam}$ in the modified capillary chemotaxis assay. Enzyme kinetic studies with crude *P. putida* ATCC 17453 lysates containing active $P450_{cam}$ (with a CO difference spectrum, **Fig S1**) under varying conditions show that 1-phenylimidazole is a competitive inhibitor of $P450_{cam}$ (**Table 2** & **Fig S9**). The toxicity of 1-phenylimidazole to *P. putida* ATCC 17453 cells was evaluated before

using it in the capillary chemotaxis assay (**Table 3**& **Fig S8**).This control assay shows that 1-phenylimidazole is neither toxic nor can be used as a carbon source by *P. putida Strain 1* (**Fig 2**).

Table 2

Camphor acted as a chemoattractant for *Strain 1* in the capillary chemotaxis assay (**Fig 3A**) using varying camphor concentrations. Since camphor has low solubility in water, we performed the capillary assays using camphor concentrations up to 1 mM. *P. putida* ATCC 17453 cells clearly chemotaxed into the syringes loaded with camphor-containing buffer, in a dose-dependent manner (**Fig 3B**). The presence of 1-phenylimidazole (a $P450_{cam}$ inhibitor) in the capillary assay prevented the chemoattraction of ATCC 17453 cells towards camphor, which is evident from the dose-dependent reduction in the number of colony forming units (CFUs) with increasing camphor concentration in the capillaries (**Fig 3C**). Therefore, the presence of active $P450_{cam}$ is essential for *P. putida* ATCC 17453 to chemotax towards camphor. Similarly, the presence of borneol, which downregulates expression of $P450_{cam}$, caused decreasing numbers of cells to be attracted to camphor in the capillary assay in a dose-dependent manner (**Fig 3D**).

The diffusion control experiments (see Methods 2.4) revealed that 1.4 ± 0.4 µM of camphor diffused from the needle into the well, and that 2.1 ± 0.1 µM of borneol diffused from the well into the needle. We did not notice any detectable amount of 1-phenylimidazole diffusing into the needle from the well. Given that all the source solutions were 1 mM, this means that 0.1% of camphor diffused out of the needle into the well, that 0.2% of borneol diffused from the well into the needle and that no detectable 1-phenylimidazole diffused from the well into the needle.

Loss of camphor by diffusion out of the needle was negligible and, therefore, the concentrations shown in Fig. 3B, as well as the constant 1 mM camphor used in the experiments with borneol and 1-phenylimidazole (Figs. 3C and 3D), are correct. Furthermore, diffusion of borneol or 1-phenylimidazole into the needle was negligible and, therefore, there could not have been confounding chemotactic effects from that.

Fig 2

Fig 3

Table 3

3.3 Survival studies with (+)-camphor and (+)-borneol

Fig 4

The toxicity of camphor and borneol against *P. putida Strains 1, 2 and 3* was investigated. Only *Strain 1* has the CAM plasmid. Survival assays were performed using varying concentrations of the test compounds (0 to 100 mM), camphor and borneol, as described in the material and methods section. Neither camphor nor borneol showed toxicity (Fig. 4A & S5) against *Strain 1*,**(Table 3**), whereas these compounds showed toxicity against *P. putida Strains2* and *3* (**Table 3**). The test compounds showed significantly higher toxicity (lower IC_{50}) to *Strain 3* compared to *Strain 2*. Among the tested compounds, camphor was more toxic than borneol towards *Strains 2* and *3*.Camphor, being a carbon source and non-toxic for *Strain 1*, became toxic in the presence of 1-phenylimidazole, a competitive inhibitor of $P450_{cam}$ (**Fig 4**, **Table 3**,& **Fig S8**).

4. Discussion

Throughout evolution, bacteria have developed a wide variety of strategies to adapt to changes in their environment. One of the adaptations to temporary changes is the ability to chemotax to spatial gradients in the concentration of a variety of chemoeffectors that can be either used as a carbon and/or an energy source. In this report, we have investigated the chemotactic ability of *P. putida* ATCC 17453 and the significant role of $P450_{cam}$ in this process.

To our knowledge, this is the first demonstration of chemotactic abilities of *P. putida* ATCC 17453 (S*train 1*). Glucose and glutamic acids are well-known bacterial attractants.[48-51] and have been included in the assay as a positive control to verify the chemotactic ability of these soil bacteria (*Strains 1, 2,* & *3*). In this report, we are trying to understand the significant role of $P450_{cam}$ in the chemotactic ability of *Strain 1* towards (+)-camphor (a carbon and energy source). In addition, we have included camphor oxidized (5-*exo*-hydroxycamphor and 5-ketocamphor) and reduced (borneol) metabolites in the chemotaxis assay to demonstrate the chemotactic behaviours they elicit.

We studied both $P450_{cam}$-induced and non-induced cultures in the chemotaxis assays. We found that non-induced cultures showed stronger chemorepulsion from borneol compared to $P450_{cam}$-induced ones. *Strains 2* & *3*, which cannot metabolize camphor (due to the absence of$P450_{cam}$), showed chemorepulsion from both camphor and borneol.

In the capillary chemotaxis assay, *Strain 1* showed chemoattraction towards camphor. To demonstrate the role of $P450_{cam}$ in the chemoattraction of *Strain 1* towards camphor, we have used 1-phenylimidazole, a known P450 inhibitor, to chemically inactivate $P450_{cam}$. We demonstrated that 1-phenylimidazole is neither attractant nor repellent within the concentration regime used in the chemotaxis assay, that this compound is neither a carbon source nor toxic to *P. putida*, and we showed in *in vitro* assays that 1-phenylimidazole is a competitive inhibitor of $P450_{cam}$. When $P450_{cam}$ activity was inhibited by 1-phenylimidazole, we noticed that *Strain 1* chemotaxed away from camphor. This is consistent with previous reports on the chemorepellent effect of inhibitors of metabolic pathways.[52-54] Similarly, when $P450_{cam}$ expression was downregulated with borneol, we have noticed that *Strain 1* showed chemorepulsion from camphor.

We have previously demonstrated that the production of borneol under low O_2 levels downregulates the expression of $P450_{cam}$ hydroxylase system,[29, 36] and thereby prevents the metabolism of camphor, an oxygen demanding process. Under these conditions of $P450_{cam}$ downregulation, *Strain 1* chemotaxed away from camphor. In previous studies on borneol production by $P450_{cam}$ in *Strain 1* we propsed that borneol acts as a signal for an insufficient level of O_2 to complete camphor degradation.[29] Here we have found that *Strain 1* bacteria chemotax away from borneol, possibly to avoid anoxic sites.

We did not notice any interfering chemotaxis in the inhibition or downregulation experiments, because both 1-phenylimidazole and borneol were (respectively) placed in the wells containing the motile cells, whereas the camphor was placed in the syringes (**Fig 3A**). If there had been an interfering chemotaxis due to the presence of borneol in the wells, we should have observed an increase in the chemoattraction towards camphor in the syringes, as bacteria move away from the chemorepellent borneol. The 1-phenylimidazole was neither attractive nor repellent at the up to 2 mM concentrations used in the capillary assay, so it also could not have been the source of interfering chemotaxis. Furthermore, diffusion of borneol or 1-phenylimidazole from the well into the needle was negligible, so this could not have caused interference either. Therefore, the

results suggest that *Strain 1* bacteria chemotax away from camphor only when functional $P450_{cam}$ is absent (either by inhibition or by downregulation of $P450_{cam}$). This is further confirmed by survival assays, where camphor being a carbon source for *Strain 1* became toxic when $P450_{cam}$ was inhibited by 1-phenylimidazole. Therefore, S*train 1* exhibits chemoattraction towards camphor only in the presence of functional $P450_{cam}$, due to the ability to metabolize camphor, like other bacterial strains that show chemotaxis towards the compounds that they can metabolize.[19, 55-59]

Bacterial chemoreception, which is necessary for bacterial chemotaxis, falls into two broad categories namely metabolism-independent (chemotaxis) and metabolism-dependent (energy taxis). Metabolism-independent chemotaxis involves the response of bacteria to gradients of chemoeffectors by sensing them through specialized transmembrane proteins called methyl accepting chemotaxis proteins (MCPs).[60] The bacteria move towards a particular direction if they detect increasing levels of chemoattractants or decreasing levels of chemorepellents. They move in the opposite direction when they sense decreasing attractant or increasing repellent levels.[61-67] Metabolism-independent chemotaxis is observed in many bacterial species, including *E. coli* and *Pseudomonas* species, towards various chemoeffectors that are not necessarily metabolized by these strains.[64-67] In contrast, metabolism-dependent energy taxis involves comparing the energy level inside the cell with the metabolism or consumption of the chemical substance that leads to a gradient in the energy level that can act as a signal.[55, 58, 59, 68-71] For metabolism-dependent chemotaxis, it is not necessary to have a specific trans-membrane receptor to recognize the ligand.[57] Such energy taxis is observed in many bacterial species, including *E. coli*, *Salmonella typhimurium*, *Azospirillum brasilense*, *Campylobacter jejuni*, *Sinorhizobium meliloti*, and *Rhodobacter sphaeroides*, etc., which show chemotaxis towards glycerol, succinate, amino acids, sugars, pyruvate, organic acids, flavones, and ammonia .[72-78]

We have noticed a gene sequence encoding an MCP on the CAM plasmid (supplementary information, **Figs. S10** & **S11**), but we do not know if it is responsible for chemoreception of camphor by *Strain 1*. The involvement of plasmid-encoded MCPs in chemotaxis has been reported in the literature.[79-82] For metabolism-dependent chemotaxis, it is not necessary to have a specific receptor to recognize the ligand.[57] Our results support the existence of

441 metabolism-dependent chemotaxis of ATCC 17453 towards camphor. However, we do not know
442 whether an additional MCP-based chemotaxis pathway is operative.
443 We also investigated the toxic effect of camphor, borneol, and 1-phenylimidazole on these
444 *Strains* (*1, 2,* & *3*), and found that none of them were toxic by themselves to *Strain 1*. However,
445 when induced *Strain 1* was treated with 1-phenylimidazole (a $P450_{cam}$ inhibitor), camphor
446 showed toxicity to this strain, because of the inability of $P450_{cam}$-inhibited strain to metabolize
447 camphor. Since cytochrome $P450_{cam}$ mediated transformation of camphor to 5-*exo*-
448 hydroxycamphor is the first and committed step in the camphor degradation pathway [83],
449 inhibition of $P450_{cam}$ in *Strain 1* bacteria cancels camphor metabolism, and non-metabolized
450 camphor is toxic to *Strain 1*. Similarly, camphor was toxic to *Strains 2* and *3*, neither of which
451 possessed $P450_{cam}$ or any camphor metabolizing ability.
452 In conclusion, we have found that *P. putida* ATCC 17453 (*Strain 1*) shows chemoattraction
453 towards its carbon source (camphor) only when functional $P450_{cam}$ is present. When $P450_{cam}$ was
454 inhibited or downregulated, it showed chemorepulsion to camphor. Investigation of the specific
455 chemotactic receptors and the ecological role of the chemoattraction of *Strain 1* towards
456 camphor should be addressed in future studies.
457

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459

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463

464 **References**

465

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Captions

Fig 1:a) Reported steps involved in the catabolic degradation of camphor by *Pseudomonas putida* (ATCC 17453). **b)** Chemical structures of (+)-borneol, (L)-glutamic acid, (D)-glutamic acid, and 1-phenylimidazole.

1= (+)-camphor; **2** = 5-*exo*-hydroxycamphor; **3** = 5-ketocamphor; **4** = unstable lactone; **5** = 2-*oxo*-Δ^3-4,5,5-trimethylcyclopentenylacetic acid; **6** = 2-*oxo*-Δ^3-4,5,5-tri-methylcyclopentenylacetyl-CoA; **7** = 3,4,4-trimethyl-Δ^3-pimelyl-CoA; **8** = (+)-borneol; **9** = (L)-glutamic acid; **10** = (D)-glutamic acid; **11** = 1-phenylimidazole; ***A*** = $P450_{cam}$, PdR, & PdX; ***B*** = 5-*exo*-hydroxycamphordehydrogenase; ***C*** = 2,5-diketocamphane monooxygenase; ***D*** = 2-oxo-Δ^3-4,5,5-trimethylcyclo-pentenylacetyl-CoA synthetase; ***E*** = 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase.

Fig 2: Effects of1-phenylimidazole (a $P450_{cam}$ inhibitor) on growth *P. putida* ATCC 17453 (*Strain 1*). **A)**Survival dose response of *Strain 1*treated with 1-phenylimidazole. **B)**Growthof *Strain 1* on minimal medium with1-phenylimidazole as a sole carbon source. Each point represents the mean ± S. E. of 3 replicates.

Fig 3: Capillary chemotaxis bioassay with (+)-camphor, 1-phenylimidazole (a $P450_{cam}$ inhibitor) and (+)-borneol (a downregulator of $P450_{cam}$ expression). **A)** Schematic representation of the capillary chemoattraction assay. The well contained the bacteria, which were either not pretreated (part **B**) or pretreated with variable concentration of 1-phenylimidazole (part **C**) or borneol (part **D**). **B)** Varying concentration of (+)-camphor and no 1-phenylimidazole.**C)** Varying concentration of 1-phenylimidazole and 1 mM (+)-camphor. **D)** Varying concentration of (+)-borneol and 1 mM (+)-camphor. Points represent the mean ± S. E. of 5 replicates. Points with different letters differ significantly ($P < 0.05$, One-way Anova followed by Tukey's post-test).

Fig 4: Survival assay with (+)-camphor in the presence and absence of 1-phenylimidazole (a cytochrome $P450_{cam}$ inhibitor).**A)** Dose response for *P. putida* ATCC 17453 treated with (+)-camphor. **B)** Dose response for *P. putida* ATCC 17453, grown in the presence of 1-phenylimidazole (20 mM) and treated with (+)-camphor. Each point represents the mean ± S. E. of 3 replicates .

Table 1: Effective concentration for 50% activity [EC_{50}] values forchemical-in-plug assays performed using the *Pseudomonas putida Strains1, 2, &3* toward selected chemoeffectors under varied conditions.

Strain[a]	Compound	Activity	Log[EC_{50}]major response			Log[EC_{50}] minor response		
			Average (mM)	95% confidence limits		Average (mM)	95% confidence limits	
				Low (mM)	High (mM)		Low (mM)	High (mM)
$P450_{cam}$ induced cultures								
Strain 1	(D)-Glucose	[b]CA	1.6	1.3	1.9			
	(L)-Glutamic acid	[b]CA	1.5	1.3	1.7			
	(L)-Glutamic acid	[c]CR				2.2	1.8	2.6
	(D)-Glutamic acid	[c]CR	2.3	1.4	3.1			
	(D)-Glutamic acid	[b]CA				~ 6.2	very wide	
	(+)-Borneol	[c]CR				~ 5.2	very wide	
	(+)-camphor with 3% Tween-20	[b]CA	1.3	0.5	2.2			
	1-phenylimidazole	[b]CA	2.2	1.9	2.4			
$P450_{cam}$ non-induced cultures								
Strain 1	(D)-Glucose	[b]CA	1.5	1.2	1.9			
	(L)-Glutamic acid	[b]CA	1.5	1.1	1.9			
	(L)-Glutamic acid	[c]CR				1.9	1.3	2.5
	(D)-Glutamic acid	[c]CR	2.7	0.6	4.8			
	(D)-Glutamic acid	[b]CA				~ 5.5	very wide	
	(+)-Borneol	[c]CR	2.8	1.7	3.9			
	(+)-camphor with 3% Tween-20	[b]CA	1.8	1.3	2.4			
Other soil bacterial cultures								
Strain 2	(+)-Borneol	[c]CR	1.3	0.9	1.7			
Strain 3	(+)-Borneol	[c]CR	2.3	1.5	3			

[a] *Strain 1 = Pseudomonas putida* ATCC 17453 (camphor-metabolizing)
Strain 2 = Pseudomonas putida ATCC 17484 (naphthalene-metabolizing)
Strain 3 = Pseudomonas putida ATCC 33015 (toluene-metabolizing)

Table 2: Enzyme kinetic analysis of crude ATCC 17453 lysates with active $P450_{cam}$.

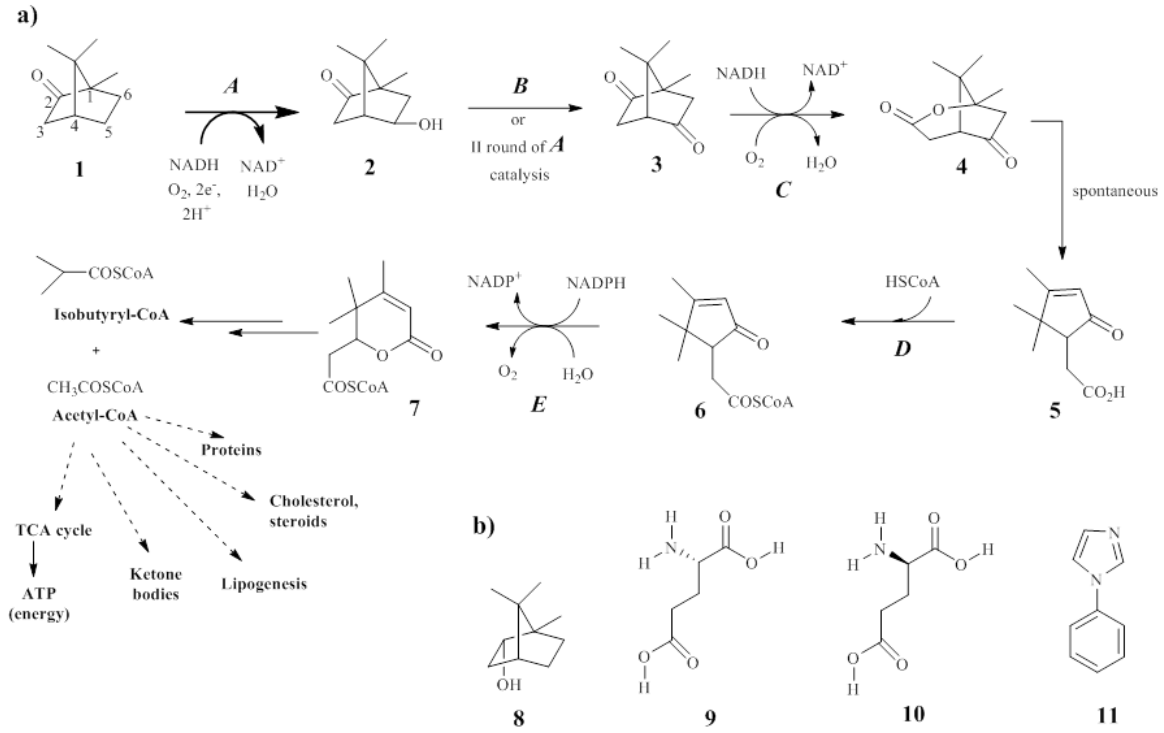
Condition	K_M (μM)	k_{cat} (min^{-1})
Varying [camphor] w/o 1-phenylimidazole	3.1 ± 1.4	0.38 ± 0.029
Varying [camphor] with 1 mM 1-phenylimidazole	72.7 ± 25.9	0.44 ± 0.033
Varying [1-phenylimidazole] with 1 mM camphor	K_i = 55.8 [a]	0.32 ± 0.04 [b]

[a] K_i determined by Lineweaver-Burk plot.

[b] This represents the minimal velocity at saturating inhibition (see Fig. S9 C).

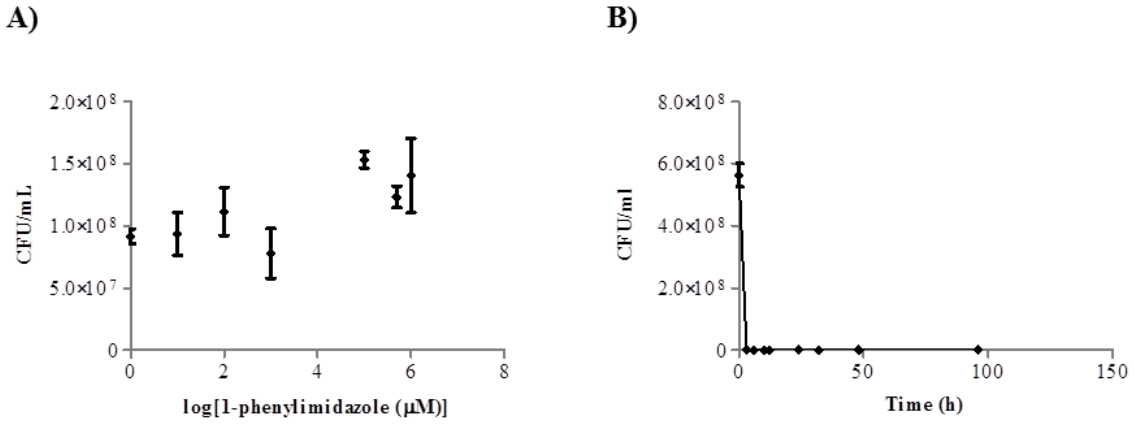
Table 3: The measured inhibitory concentration at 50% activity [IC_{50}] values for the survival assays performed using different compounds on the selected soil bacteria.

Bacterial Strain	Compound	Activity	IC_{50} (mM)
ATCC 17453 (*Strain 1*)	(+)-Camphor	Non-toxic	-
	(+)-Borneol	Non-toxic	-
	1-Phenylimidazole	Non-toxic	-
	(+)-Camphor in the presence of 1-phenylimidazole	Toxic	2.0
ATCC 17484 (*Strain 2*)	(+)-Camphor	Toxic	20.0
	(+)-Borneol	Toxic	50.1
ATCC 33015 (*Strain 3*)	(+)-Camphor	Toxic	2.0
	(+)-Borneol	Toxic	2.5



721

722 Figure 1.



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724 Figure 2.

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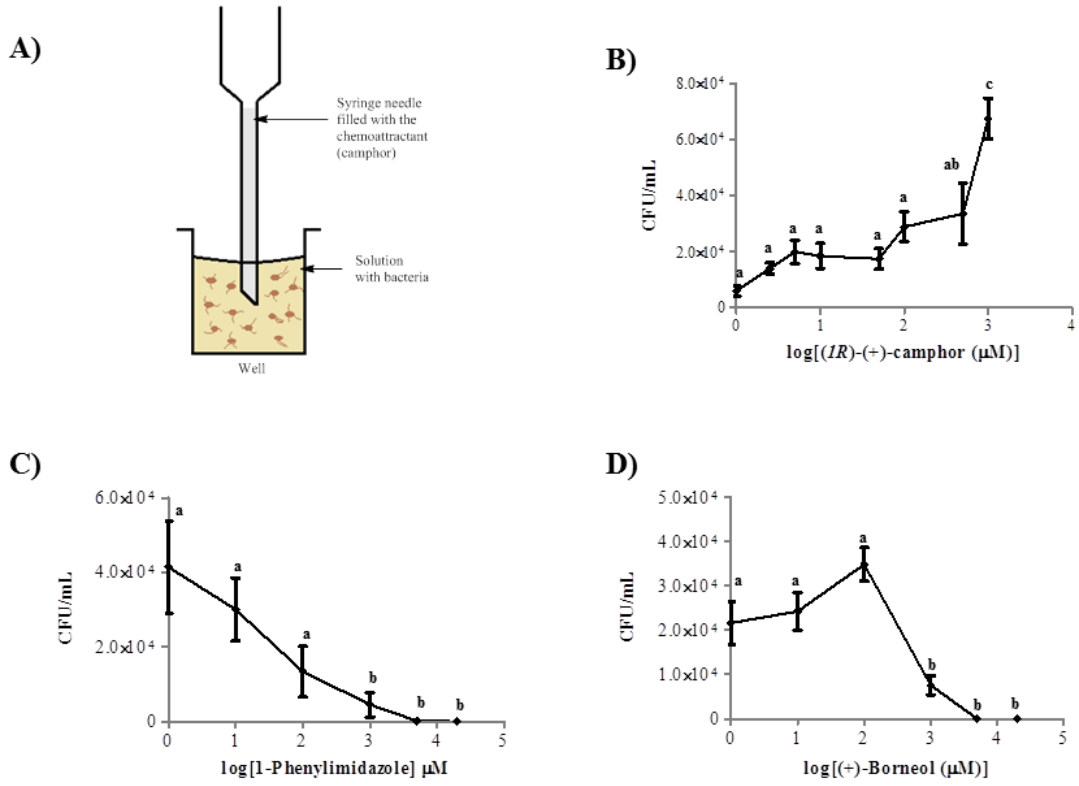

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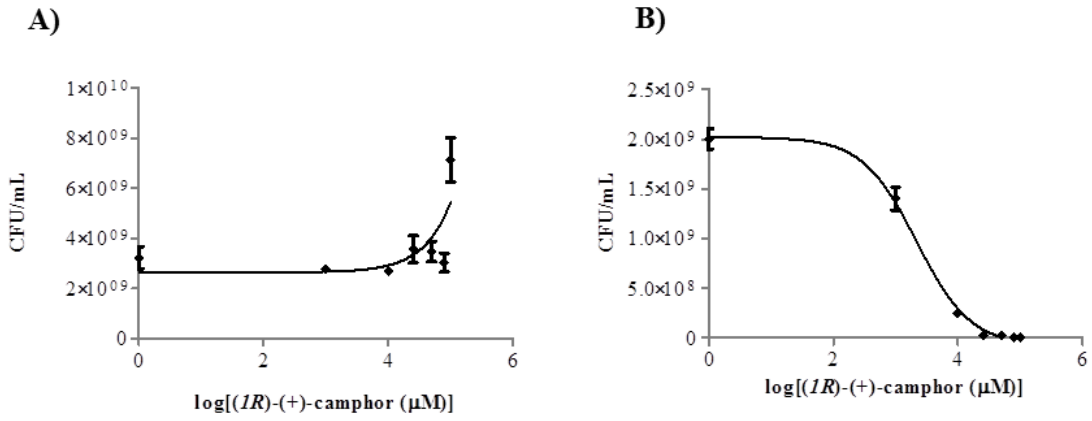
729 Figure 3.



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731 Figure 4.

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