JOURNAL OF BACTERIOLOGY, July 1989, p. 4063–4066
0021-9193/89/074063-04$02.00/0

Vol. 171, No. 7

NOTES

Flagellation of *Pseudomonas putida* and Analysis of Its Motile Behavior

CAROLINE S. HARWOOD,[1]* KATHY FOSNAUGH,[2] AND MARILYN DISPENSA[1]

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242,[1] and Department of Biology, University of California San Diego, La Jolla, California 92093[2]

Received 5 December 1988/Accepted 27 March 1989

***Pseudomonas putida* flagella were examined. Also, changes in motile behavior in response to chemoattractants were analyzed quantitatively by computer. Reversals in the rotation direction of bundles of polar flagella resulted in changes in swimming direction. Cells swimming in buffer changed direction once every 2 s on average, whereas cells exposed to the attractant benzoate changed direction an average of once every 10 s. The findings show that *P. putida* responds to temporal gradients of chemoattractant by suppressing changes in the direction of rotation of flagella.**

Aromatic compounds are a major component of plants and also constitute a substantial proportion of the toxic wastes that are released into the environment (3, 5). Many kinds of bacteria can grow on various aromatic compounds, and several groups of motile soil bacteria, including *Pseudomonas*, *Rhizobium*, and *Agrobacterium* spp., can also sense and respond behaviorally to the presence of aromatics in the environment (1, 8, 17, 18). Since chemotaxis can be viewed as an important prelude to metabolism, an understanding of mechanisms underlying responses to aromatic compounds has practical implications, for example, for strategies aimed at using bacteria to detoxify polluted environments. Also, chemotaxis could be an important factor guiding the association of rhizobacteria with plant roots, sites expected to be rich in aromatic material.

Pathways of aromatic acid metabolism in *Pseudomonas putida* have been studied particularly intensively, which makes this a good organism to use for the study of behavioral responses to aromatic compounds. *P. putida* synthesizes at least two distinct chemoreceptors for aromatic acids, a benzoate chemoreceptor [induced when cells are exposed to β-ketoadipate (a metabolite of benzoate and *p*-hydroxybenzoate)] and a benzoylformate chemoreceptor [induced by L(+) mandelate or benzoylformate (8)]. To date, the chemotactic behavior of *P. putida* has been analyzed by measuring the accumulation of cell populations in capillaries in response to spatial gradients of aromatic compounds. Although the capillary assay provides a direct measure of chemotaxis, this technique is time-consuming and provides no information about changes in the motile behavior of individual cells that occur in response to chemical stimuli. Such information is required to fully understand how cells process sensory stimuli from aromatic compounds to effect chemotactic responses. In this paper we describe a computer program that we developed to quantitatively analyze the motile behavior of individual *P. putida* cells. We also investigated the arrangement and operation of flagella in this organism, since only very limited information was available about fundamental aspects of *P. putida* motility.

* Corresponding author.

P. putida PRS2000 (wild type) (16) was cultivated in defined mineral medium as described previously (8).

Cells for light-microscopy observations and videomicroscopy were harvested by centrifugation in the early to mid-logarithmic phase of growth and suspended in chemotaxis buffer (50 mM potassium phosphate [pH 7.0], 10 μM EDTA) to a density of 4×10^6 to 6×10^6 cells per ml. Approximately 80 to 90% of the cells were motile for at least 1 h.

Cells for electron microscopy were harvested in the early to mid-logarithmic phase of growth and suspended in distilled water to a concentration of 10^8/ml. Cell suspensions were dried onto Formvar-coated grids and shadowed with platinum-palladium (80 and 20%, respectively) at an angle of 20° before examination with a Philips 300 transmission electron microscope operating at 80 kV.

High-intensity illumination dark-field microscopy observations were made by using a 450-W xenon short-arc lamp and an oil immersion dark-field condenser, as described previously (13).

For quantitative analyses of motility, an IM-35 inverted dark-field microscope fitted with a 40 × neofluar objective (Carl Zeiss, Inc.) was used to observe cells in a 10-μl suspension placed on a glass cover slip. Cells were illuminated with a 12-V, 60-W tungsten lamp; the light was passed through a heat reflection filter. A video camera (series 67; Dage-MTI, Inc.) was mounted on the microscope, and images were recorded with a Panasonic PV8950 video recorder. A computerized cell-tracking system (Motion Analysis Systems Inc., Santa Rosa, Calif.) was used to analyze the motility of videorecorded cells. The Motion Analysis system digitizes video images frame by frame and connects the centers of these images to form paths. The system also calculates the rate of change of direction (the absolute value of the angular velocity) and the speed at each point in the path of a cell. The motion of cells in paths was analyzed with a user program (BOP) designed to study the behavior of *P. putida*. This was based on similar programs that have used the Motion Analysis system to examine the motile behavior of other bacteria (4, 21). Tape segments (5 s long) of videorecorded cells were fed into the computer at a rate of 30 frames per s. Cell paths of various lengths were analyzed depending on how long cells stayed in focus or in the field of

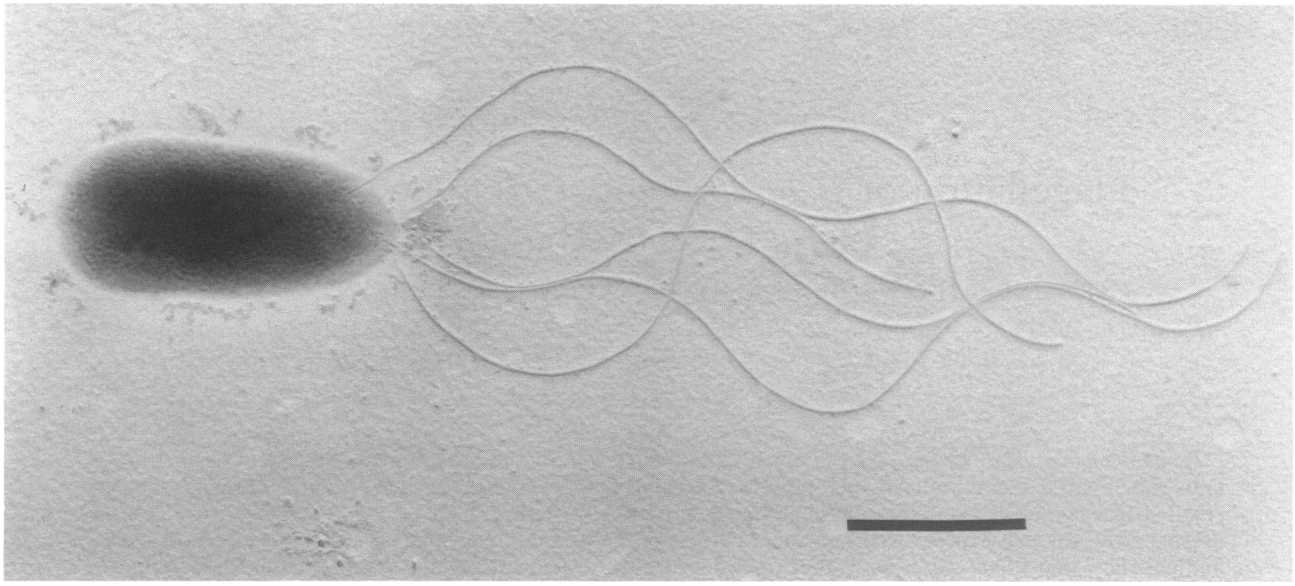

FIG. 1. Electron micrograph of *P. putida* PRS2000. Bar, 1 μm.

view. Cells with a path length of less than 30 μm were not analyzed. Paths of cells with a minimum speed of less than 1.5 μm/s or an average speed of less than 17 μm/s were also eliminated. Thus, cells which were nonmotile or which stuck to the cover slip were not included in the analysis. Cells were defined as smooth-swimming when they had a speed of >10.65 μm/s and a rate of change of direction of <700°/s. Cells were defined as undergoing a change of direction when their speed dropped to <10.65 μm/s and the rate of change of direction increased to >700°/s. These values were determined empirically; the number of changes of direction computed with BOP correlated very closely with the number of changes of direction observed visually. False changes of direction were sometimes generated when two paths crossed. To minimize this problem, we used a low cell density.

Chemical stimuli (in 1-μl volumes) were added to 9-μl volumes of cell suspensions immediately preceding the videotaping. To avoid analyzing cells that had started to adapt to the stimulus, the stimulated cells were videotaped within 5 to 30 s. Adaptation experiments involved videotaping the cells for 2 to 4 min and analyzing 5-s segments periodically. Responses to benzoate were tested by using cells that had been grown with *p*-hydroxybenzoate as the sole carbon and energy source. Growth on this compound elicits the benzoate chemotactic response but not benzoate metabolism.

Flagellation of *P. putida* cells. *P. putida* has been reported to have multiple polar flagella (12, 20). However, no ultrastructural examinations of this species have been reported, and precise information about the flagellation of strain PRS2000 was also not available. We therefore examined *P. putida* PRS2000 cells by electron microscopy and found that cells generally had between five and seven flagella inserted at one end to form a tuft (Fig. 1). Occasionally, cells with as few as 1 and as many as 12 flagella were seen. The flagellar filaments had a conventional waveform and were usually 2 to 3 wavelengths long.

Motile behavior of *P. putida*. When free-swimming cells were videotaped and the paths of individual cells were tracked by computer-assisted motion analysis, the resulting qualitative picture of motile behavior consisted of unidirectional swimming punctuated by abrupt changes in direction (Fig. 2A). Changes in direction were completed within 20 to 30 ms and were not accompanied by the tumbling motion

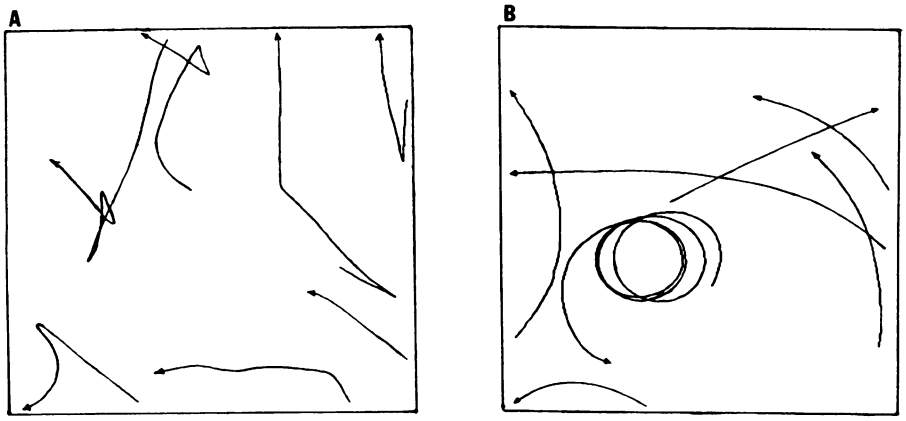

FIG. 2. Examples of computer-generated paths of *P. putida* cells in chemotaxis buffer 5 s after addition of chemotaxis buffer (A) or benzoate (500 μM, final concentration) (B).

TABLE 1. Quantitative analysis of *P. putida* behavior[a]

Expt no.[b]	No. of paths	Duration of behavior (s)	No. of changes of direction	
			Total	Avg/s[c]
1	35	35	18	0.51
2	83	91	38	0.42
3	59	73	53	0.72
4	116	141	46	0.33
5	110	128	39	0.30
6	73	73	32	0.44
7	120	120	68	0.57
8	39	46	45	0.98

[a] Behavior was analyzed with the BOP program and computer-assisted motion analysis as described in the text.
[b] Data for each experiment were collected on different days with separately grown cultures of cells.
[c] The mean change of direction when each second of behavior was equally weighted was 0.50/s.

typical of peritrichously flagellated bacteria such as *Escherichia coli* and *Salmonella typhimurium*. Sometimes, instead of abruptly changing the direction of forward swimming, cells swam backwards for a short distance before they resumed swimming smoothly in a forward direction. Bundles of flagella about two body lengths long could be seen trailing smooth-swimming cells that were viewed under high-intensity dark-field illumination. These bundles disappeared as the cells changed swimming direction.

Smooth-swimming *P. putida* cells placed on the surface of a glass cover slip moved in a counterclockwise (CCW) direction when viewed from below with an inverted microscope (Fig. 2B). Curved paths have been observed with other motile bacteria (7, 22) and are due to frictional resistance between rotating cell bodies and the glass surface. Cell trajectories in the CCW direction would be expected to occur as a result of *P. putida* flagellar rotation in the CCW direction. Smooth-swimming *E. coli* cells were also observed to move in a CCW direction as viewed with our microscope setup.

These observations are consistent with a view of *P. putida* flagellar behavior that is similar to *E. coli* and *S. typhimurium* behavior; flagellar bundles rotating in the CCW direction are responsible for smooth swimming, and a reversal in the rotation direction of the flagellar motors causes the bundles of flagella to fly apart and the cells to change direction. In the case of the peritrichously arranged flagella of the enteric bacteria, changes in the direction of rotation are accompanied by a tumbling motion of the cell body (11, 14, 19). The polar insertion of flagella in *P. putida* probably accounts for the observed reorientation of the cell body without tumbling. Smooth swimming would resume as flagellar motors reversed for a second time to allow reformation of a CCW-rotating flagellar bundle. The backwards swimming of *P. putida* cells can be accounted for by a synchronous reversal of flagellar motors. This would result in the propagation of a negative waveform from the flagellar bundle (14). The reversal of a single polar flagellum would also result in backward swimming (22).

Computer analysis of behavior in response to chemoattractants. When populations of cells were analyzed by computer-assisted motion analysis, behavior could be quantitatively assessed in terms of the average number of changes of direction of swimming per second (Table 1). For cells suspended in chemotaxis buffer this was 0.50/s. Addition of the chemoattractant benzoate to suspensions of *P. putida* cells that had been grown on *p*-hydroxybenzoate (so that synthesis of the benzoate chemoreceptor was induced) resulted in a drastic modification of behavior; cells changed swimming direction much less frequently and swam smoothly for prolonged periods (Fig. 2). The average number of changes of direction per second upon addition of 500 μM benzoate was 0.10. The inference that the smooth-swimming response of *P. putida* cells to benzoate addition is related to chemotaxis was borne out by experiments with cells grown on carbon sources (e.g., glucose, acetate, or arginine) that had previously been shown not to induce benzoate taxis as measured in capillary assays. Such cells did not modify their behavior in response to benzoate addition in temporal assays (data not shown). Furthermore, as with the responses of cells to spatial gradients of attractants (8), there was a concentration dependence of the response of *P. putida* cells to temporal gradients of benzoate (Fig. 3). The smooth-swimming response was maximal at benzoate concentrations above about 100 μM and was not detectable at benzoate concentrations below 5 to 10 μM.

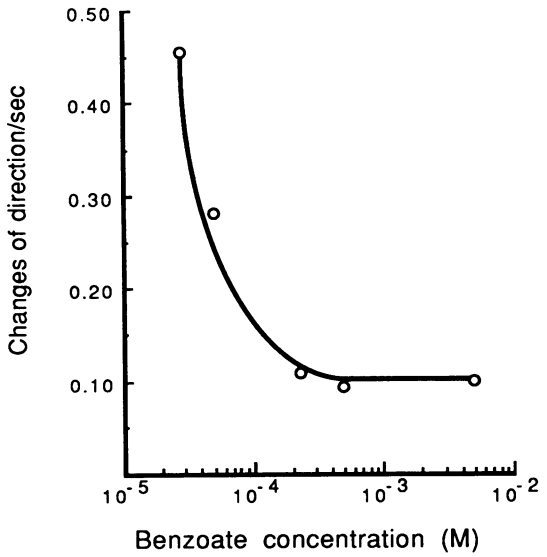

FIG. 3. Responses of *P. putida* cells to additions of various concentrations of benzoate. Each point is the average of values obtained from individual cultures of cells. At least three separate cell cultures were analyzed for each point, and at least 30 s of behavior was included in each analysis.

The average linear speed of smooth-swimming cells either after buffer addition or after addition of 500 μM benzoate was 44 μm/s. Individual cells reached speeds of up to 75 μm/s. Addition of benzoate to very high concentrations (50 mM, pH 7.0) resulted in a decrease in the average linear speed of smooth-swimming cells to 26 μm/s, but the smooth-swimming response still occurred, and stimulated cell populations had an average direction change rate of 0.07/s. The swimming speed of glucose-grown cells also decreased markedly upon addition of 50 mM benzoate, but the frequency of changes of direction remained unaltered from the prestimulus level. A probable explanation for these observations is that benzoate, a weak acid, is able to either partially or fully collapse the transmembrane proton gradient when present at high concentrations (10). A resulting reduced proton motive force would then be expected to lead to a decrease in flagellar motor speed. Unlike other bacteria that have been examined (9), this does not appear to affect the direction-switching probabilities of the flagellar motors of *P. putida*.

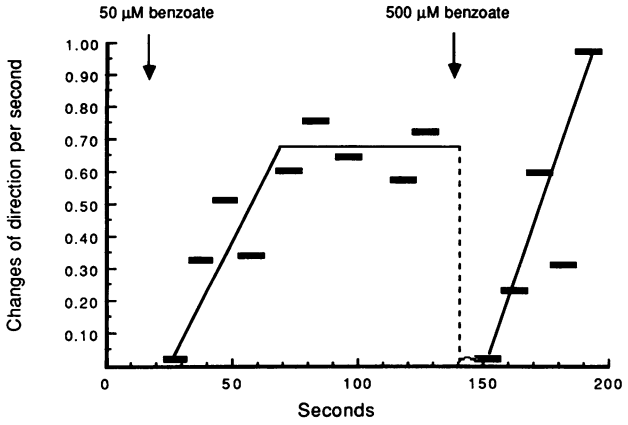

FIG. 4. Effect of benzoate on the motile behavior of a population of *P. putida* cells. Cells were stimulated with 50 and 500 μM (final concentrations) benzoate at the points indicated. The horizontal bars indicate the time interval (5 s) for each analysis.

The change in cell behavior in response to benzoate addition was temporary. Cells adapted and returned to their prestimulus behavior within 1 to 2 min and could be stimulated again by further addition of a higher concentration of benzoate (Fig. 4). This response resembles those of other bacteria to temporal gradients of attractants. Excitation is rapid, the stimulated cells exhibit smooth swimming, and then they adapt to the stimulus within a few minutes (2, 4, 6, 15).

The behavioral responses of cells to other chemicals in addition to benzoate also reflected chemotactic responses, as measured in capillary assays. For example, cells showed a strong response to Casamino Acids (Difco Laboratories, Detroit, Mich.) and to succinate in both temporal and capillary assays, and benzoylformate elicited a temporal response from *P. putida* cells that had been grown on benzoylformate (data not shown).

These results show that the BOP computer program that we have designed for use in conjunction with an automated motion analysis system can be used to measure changes in the behavior of *P. putida* cells that occur in response to additions of chemicals. Furthermore, the results of the temporal assay of *P. putida* chemotactic behavior agree with those of the capillary assay. The temporal assay is much less time-consuming than the capillary assay and is also more sensitive. The responses of cells to a temporal addition of a strong chemoattractant such as benzoate are so dramatic that simple visual inspection can be used to obtain a qualitative impression of whether or not a smooth-swimming response has occurred. Thus, it is possible, for example, to screen a large number of strains to identify those that are likely to be chemotaxis mutants.

Except for a few details which can be attributed mainly to differences in flagellar arrangement, the motile behavior of *P. putida* appears to be very similar to that of the enteric bacteria *E. coli* and *S. typhimurium*. However, *P. putida* responds to a very different repertoire of chemoattractants.

We thank E. Peter Greenberg for helpful discussions and for the use of the Motion Analysis System and Charlotte Kämpf for her extensive assistance in programming the operating system of the computer. We thank Rebecca Parales for help with the high-intensity dark-field microscopy observations and Robert Macnab for the use of equipment and for his help in interpreting the observations. We thank Rhea Garen and William Ghiorse for help with the electron microscopy.

This work was supported by Public Health Service grant GM35086 from the National Institutes of Health.

LITERATURE CITED

1. **Ashby, A. M., M. D. Watson, G. J. Loake, and C. H. Shaw.** 1988. Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C[1] toward *vir*-inducing phenolic compounds and soluble factors from monocotyledenous and dicotyledonous plants. J. Bacteriol. **170:**4181–4187.
2. **Berg, H. C., and D. A. Brown.** 1972. Chemotaxis in *Escherichia coli* analysed by three-dimensional tracking. Nature (London) **239:**500–504.
3. **Crawford, R. L.** 1981. Lignin biodegradation and transformation. John Wiley & Sons, Inc., New York.
4. **Fosnaugh, K., and E. P. Greenberg.** 1988. Motility and chemotaxis of *Spirochaeta aurantia*: computer-assisted motion analysis. J. Bacteriol. **170:**1768–1774.
5. **Ghisalba, O.** 1983. Microbial degradation of chemical waste, an alternative to physical methods of waste disposal. Experientia **39:**1247–1257.
6. **Goldman, D. J., and G. W. Ordal.** 1981. Sensory adaptation and deadaptation by *Bacillus subtilis*. J. Bacteriol. **147:**267–270.
7. **Götz, R., and R. Schmitt.** 1987. *Rhizobium meliloti* swims by unidirectional, intermittent rotation of right-handed flagellar helices. J. Bacteriol. **169:**3146–3150.
8. **Harwood, C. S., M. Rivelli, and L. N. Ornston.** 1984. Aromatic acids are chemoattractants for *Pseudomonas putida*. J. Bacteriol. **160:**622–628.
9. **Khan, S., and R. M. Macnab.** 1980. The steady-state counterclockwise/clockwise ratio of bacterial flagellar motors is regulated by protonmotive force. J. Mol. Biol. **138:**563–597.
10. **Kihara, M., and R. M. Macnab.** 1981. Cytoplasmic pH mediates pH taxis and weak-acid repellent taxis of bacteria. J. Bacteriol. **145:**1209–1221.
11. **Larsen, S. H., R. W. Reader, E. N. Kort, W.-W. Tso, and J. Adler.** 1974. Change in direction of flagellar rotation is the basis of the chemotactic response in *Escherichia coli*. Nature (London) **249:**74–77.
12. **Lautrop, H., and O. Jessen.** 1964. On the distinction between polar monotrichous and lophotrichous flagellation in green fluorescent pseudomonads. Acta Pathol. Microbiol. Scand. **60:** 588–598.
13. **Macnab, R. M.** 1976. Examination of bacterial flagellation by dark-field microscopy. J. Clin. Microbiol. **4:**258–265.
14. **Macnab, R. M.** 1977. Bacterial flagella rotating in bundles: a study in helical geometry. Proc. Natl. Acad. Sci. USA **74:** 221–225.
15. **Macnab, R. M., and D. E. Koshland, Jr.** 1972. The gradient-sensing mechanism in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **69:**2509–2512.
16. **Ornston, L. N., and D. Parke.** 1976. Properties of an inducible uptake system for β-ketoadipate in *Pseudomonas putida*. J. Bacteriol. **125:**475–488.
17. **Parke, D., L. N. Ornston, and E. W. Nester.** 1987. Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. J. Bacteriol. **169:**5336–5338.
18. **Parke, D., M. Rivelli, and L. N. Ornston.** 1985. Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. J. Bacteriol. **163:**417–422.
19. **Sager, B. M., J. J. Sekelsky, P. Matsumura, and J. Adler.** 1988. Use of a computer to assay motility in bacteria. Anal. Biochem. **173:**271–277.
20. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
21. **Sundberg, S. A., M. Alam, and J. L. Spudich.** 1986. Excitation signal processing times in *Halobacterium halobium*. Biophys. J. **50:**895–900.
22. **Taylor, B. L., and D. E. Koshland, Jr.** 1974. Reversal of flagellar rotation in monotrichous and peritrichous bacteria: generation of changes in direction. J. Bacteriol. **119:**640–642.