Interactions between *Caenorhabditis elegans* Individuals during Chemotactic Response

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ABSTRACT—The chemotactic response of the nematode *Caenorhabditis elegans* is known to be affected by the population density on an assay plate, suggesting the existence of interactions between individual animals. To clarify the interactions between individuals during chemotaxis, we investigated the effect of population density at an attractant area on the chemotactic response to water-soluble sodium acetate and odorant diacetyl using wild-type N2 animals and *daf-22 (m130)* mutants, which have defective pheromone secretion but can sense pheromone. The chemotaxis index of N2 animals at 90 min of the assay negatively correlated with the number of animals on the assay plate regardless of the type of attractant used ($p < 0.01$). On the other hand, there was no significant difference in the chemotaxis indices of *daf-22 (m130)* mutants for either of the attractants between the low- and high-population groups. When *daf-22 (m130)* mutants of a high population density were placed at the attractant location in advance, the chemotaxis index of N2 animals was almost the same as that in the control assay in which no animals were placed at the attractant location in advance. When N2 animals of a high population density were placed at the attractant location in advance, the chemotaxis indices of N2 animals and *daf-22 (m130)* mutants were significantly smaller than those obtained in the control assay ($p < 0.05$). In the absence of an attractant, we observed a decline in the fraction of animals in the neighborhood of N2 animals of a high population density, although the nematodes were not influenced by *daf-22 (m130)* mutants of a high population density. These results suggest that the attraction of nematodes to chemicals is inhibited by an increase in the concentration of the pheromone generated by N2 animals at the attractant location.

Key words: *Caenorhabditis elegans*, chemotaxis, diacetyl, pheromone, population density, sodium acetate

INTRODUCTION

The nematode, *Caenorhabditis elegans*, lives in soil and at air and water interfaces, and feed on soil bacteria (Andrew and Nicholas, 1976). It shows a chemotactic response to various compounds including some ions (*e.g.*, Na^+ and Cl^-), amino acids, alcohols and other substances, which are produced by food bacteria and act as natural attractants of chemotaxis (Ward, 1973; Bargmann and Horvitz, 1991; Bargmann *et al.*, 1993). The presentation of chemical stimuli such as water-soluble substrates (*e.g.*, NaCl and lysine) and volatile odorants (*e.g.*, diacetyl and isoamyl alcohol) elicits an attractive response of the nematode (Ward, 1973; Dusenbery, 1974; Bargmann *et al.*, 1993), and presentation of chemical stimuli such as Cu^{2+} , SDS and benzaldehyde at high concentrations elicits an

avoidance response (Bargmann *et al.*, 1993; Nuttly *et al.*, 2001; Hilliard *et al.*, 2002). These chemicals are sensed by chemosensory neurons in the head sensory organ amphid and the tail organ phasmid (Ward, 1973; Bargmann and Horvitz, 1991; Hilliard *et al.*, 2002), and information on these chemicals is transmitted to several interneurons and play a significant role in mediating chemotactic behavior.

In chemotaxis assays for nematodes, many animals are generally used in one assay plate to evaluate the fraction of animals that gather at an attractant area; this fraction is known as the chemotaxis index (Bargmann *et al.*, 1993; Matsuura *et al.*, 2004a). However, the relationship between the index and population of nematodes on an assay plate was not clarified. Recently, a difference in the time of aversive response to 100% benzaldehyde between groups and individually tested animals was observed (Nuttly *et al.*, 2001). Moreover, in a preliminary report, we showed decreases in chemotaxis indices for sodium acetate and diacetyl with an increase in the number of wild-type animals on an

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assay plate (Matsuura *et al.*, 2004b). Although these observations indicate the existence of interactions between individuals during population assays, the regulation mechanism of chemotactic response of nematodes has not yet been clarified.

It is known that the dauer pheromone is produced by all nematodes, and its concentration is used by these animals as a measure of population density (Golden and Riddle, 1984). *C. elegans* exhibits developmental selection in its life cycle which is influenced by environmental conditions. Under favorable conditions, its life cycle consists of four larval stages. If environmental conditions are unfavorable, nematodes may have arrested development following the L2 stage and become a dauer larva which is well adapted for long-term survival and is tolerant to harsh conditions (Riddle and Albert, 1997). The selection between development into normal and dauer larvae is modulated by food availability, temperature and dauer pheromone concentration (Riddle and Albert, 1997; Ailion and Thomas, 2000). It is possible that an increase in pheromone concentration with population density affects the behavior of *C. elegans* during chemotaxis.

The aim of the present study was to investigate the effect of population density on the chemotactic response of nematodes and to clarify the mechanism of interactions between individual animals. First, we examined the chemotactic response of wild-type N2 animals and *daf-22(m130)* mutants, which were defective in pheromone secretion (Golden and Riddle, 1985), to water-soluble sodium acetate and odorant diacetyl under the condition of various populations. Then, we investigated the chemotactic responses of the nematodes under the condition in which N2 animals or *daf-22(m130)* mutants of a high population densities were placed at the attractant location in advance to clarify the effect of pheromone on chemotactic response. The effect of pheromone on the animal's behavior was also examined in the absence of an attractant. The data of the present study may possibly lead to a better understanding of the principle of communication between nematodes.

MATERIALS AND METHODS

Animals

Caenorhabditis elegans wild-type strain (Bristol N2) and *daf-22(m130)* mutants, which were defective in pheromone secretion, were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. The nematodes were grown and maintained on NGM agar plates (3 g/l NaCl, 2.5 g/l polypeptone, 5 mg/l cholesterol, 1 mM CaCl₂, 1 mM MgSO₄, 25 mM KH₂PO₄ (pH 6.0) and 17 g/l agar) with the *Escherichia coli* OP50 strain in an incubator (SANYO MIR253) at 20°C (Brenner, 1974). In all experiments, we used synchronously staged young adult hermaphrodites of N2 animals obtained approximately 70 hr after hatching at 20°C. We also used young adult hermaphrodites of *daf-22(m130)* mutants obtained approximately 75 hr after hatching at 20°C, because the development of *daf-22(m130)* mutants was more delayed in comparison with that of N2 animals.

Chemotaxis assays

In all chemotaxis assays, we used 9-cm-diameter tissue culture dishes for assay containing 1 mM CaCl₂, 1 mM MgSO₄, 5 mM KH₂PO₄ (pH 6.0) and 15 g/l agarose, in which NaCl was omitted. The nematode showed the highest attraction to 1.0 M sodium acetate (CH₃COONa) and 0.1% diacetyl in our previous study (Matsuura *et al.*, 2004a). Therefore, we used these concentrations for the attractants in chemotaxis assays. Sodium acetate (7 µl) was placed twice on the surface of an assay plate (attractant location; location A in Fig. 1A) 15~18 hr and 3 hr before the start of the experiment to obtain a concentration gradient for the assay of sodium acetate. The assay plate was placed in an incubator at 20°C. To anesthetize the animals, 1 µl of 0.5 M sodium azide was spotted at the same location as that of sodium acetate shortly before the experiment. As a control, 1 µl of 0.5 M sodium azide was also spotted 4 cm away from the attractant location (control location; location B in Fig. 1A). For the assay of diacetyl, 1.5 µl of diacetyl dissolved in 99.5% ethanol and 1 µl of 0.5 M sodium azide were spotted at location A (attractant location, Fig. 1A) of an assay plate shortly before the experiment. As a control, 1.5 µl of 99.5% ethanol and 1 µl of 0.5 M sodium azide were spotted at a control location (location B in Fig. 1A).

Fig. 1. Configuration of assay plate. **(A)** Chemotaxis to attractants. Nematodes were placed in two locations (C and D) equidistant (approximately 2.8 cm) from the attractant and control spots (A and B). For the assay of either sodium acetate or diacetyl, 7 µl of 1.0 M sodium acetate (Na) or 1.5 µl of 0.1% diacetyl was spotted at the center of the attractant location. To anesthetize the animals, 1 µl of 0.5 M sodium azide was spotted at the attractant and control locations. The numbers of animals at both locations (A and B) were determined every 10 min over a 90-min period. **(B)** Assays for substance produced by nematodes. Approximately 30 animals marked with Indian ink were placed at the midline of the assay plate. A 7-mm-radius circular plug of agarose containing 100 animals was placed at location B in the test area. The plug in the absence of animals was also placed at location A in the control area. The numbers of animals at the test and control areas were determined for 60 min.

Nematodes were washed in a 15-ml conical centrifuge tube with buffer (1 mM CaCl₂, 1 mM MgSO₄, 5 mM KH₂PO₄ (pH 6.0), and 0.05% Tween 20) after removal from the growth plates with bacteria as food, and centrifuged for 20 sec at 600 rpm. All the animals were washed three times prior to use. The nematodes were then aspirated from the bottom of the tube using a micropipette (3 μ l) and placed at two locations (C and D) equidistant (2.8 cm) from the attractant and control spots (Fig. 1A). The animals from both locations were allowed to dry, and the clumped animals were separated from each other by gently touching them with a Kimwipe and then left to move freely on the assay plate for 90 min. The numbers of animals at the attractant and control locations A and B (circle of 1 cm radius) were determined under a microscope (OLYMPUS SZ40) every 10 min. All experiments were performed during day-time at 20°C.

To determine the effect of population density at an attractant location on chemotaxis, nematodes that gathered at the attractant location of sodium acetate were removed every 10 min or at 40 min. In this experiment, we used approximately 100 animals per plate. A control assay, in which the lid of the plate was opened but the nematodes were not removed from the attractant location, was also performed under the condition of a high population density (approximately 100 animals). The total number of animals that gathered and were removed from the attractant location was determined for a 90-min period.

The effect of a high population density at an attractant location on chemotaxis was also investigated. Approximately 150 animals were placed at the two original locations and then gathered at the attractant location of 1.0 M sodium acetate or 0.1% diacetyl for 60 min in advance. When the number of animals at the attractant location were fewer than 100, some nematodes at the other locations were gently moved to the attractant location using a platinum wire (0.1 mm diameter). Then approximately 30 pellet animals marked with Indian ink were placed at the original locations (C and D), and their chemotactic responses were observed for 90 min. To mark the assay nematodes, these animals were given food (*E. coli*) with Indian ink for 3 hours before the assay. No difference in chemotactic response between the marked and nonmarked nematode groups was observed (data not shown).

Assays for substance produced by nematodes

To examine whether the substance produced by nematodes, *i.e.*, a pheromone, affects the behavior of animals as repellent, the responses of nematodes to N2 animals and/or *daf-22 (m130)* mutants of a high population density were tested in the absence of an attractant. First, approximately 100 animals were gathered at the location of diacetyl for 60 min. In this case, 1.3 μ l of 0.01% diacetyl was placed at the lid of the plate. To anesthetize the animals, 1 μ l of 0.5 M sodium azide was spotted on the surface of the plate. Then the lid of the plate was opened, and the plate was left for 60 min in air to remove an efficacy of diacetyl. After that, a 7-mm-radius circular plug of agarose was removed from another plate (assay plate), and the same size of plug of agarose containing approximately 100 animals was placed in the space where the agarose had been removed (location B in the test area; Fig. 1B). The plug, which was pre-exposed to 0.01% diacetyl in the absence of nematodes, was also placed at an opposite location (location A in the control area; Fig. 1B). The space between the assay plate and the plug was filled with agarose.

Nematodes marked with Indian ink were washed three times, and approximately 30 animals were placed at the midline of the assay plate (Fig. 1B). The preparation of this experiment took 2 hr. When the duration of assay was more than 60 min, some animals came out of anesthesia. Therefore, the numbers of animals at the test and control areas were determined under a microscope for 60 min in this assay (Fig. 1B). Nematodes that remained within 1 cm from the midline (original area) were not counted, because animals

accidentally wandered into either the test and control areas.

Scoring and analysis

In chemotaxis assays (Fig. 1A), the chemotaxis index was calculated based on the number of animals that gathered at the attractant location (Bargmann *et al.* 1993). In the assays for the substance produced by nematodes (Fig. 1B), the population index and fraction of animals were also calculated using the following equation:

Chemotaxis index = (number of animals at attractant location – number of animals at control location) / total number of animals on plate.

Population index = (number of animals in test area – number of animals in control area) / total number of animals on plate.

Fraction of animals = number of animals in test area or control area / total number of animals on the plate.

The chemotaxis and population indices showed a positive value when the nematodes elicited the attractive response, while the indices were less than zero when the nematodes showed an avoidance response. The correlation coefficient between the chemotaxis index and the total number of animals on a plate was analyzed using statistical software (Microsoft Excel 2001). The significance of difference in chemotaxis index was statistically analyzed by Student's *t*-test. Values are presented as means $\pm$ S.E.M. A minimum of ten assays were performed for each series of experiments.

RESULTS

Change in chemotaxis index by population density at attractant location

To determine the effect of population on chemotactic response to an attractant, the chemotactic indices of N2 animals to a water-soluble attractant (sodium acetate) and a volatile odorant (diacetyl) at various population densities were examined. Under both conditions of low (approximately 30 animals on the assay plate) and high (approximately 100 animals on the assay plate) populations, the chemotaxis index for 1.0 M sodium acetate increased rapidly within 40 min and then continued to increase gradually during the assay. However, the value under the condition of a high population was significantly lower than that under the condition of a low population ($p < 0.05$) (Fig. 2A). The chemotaxis indices for sodium acetate under low and high populations at 90 min were 0.84 ± 0.03 and 0.64 ± 0.05 , respectively. As shown in Fig. 2B, a negative correlation between the chemotaxis index at 90 min and the number of animals on the plate was observed ($r = 0.57368$, $p < 0.001$).

Under both conditions of low and high populations, the chemotaxis index for 0.1% diacetyl increased rapidly within 40 min and then continued to increase gradually during the 90 min of assay (Fig. 2C). Similar to the response to sodium acetate, the index under a high population was lower than that under a low population, and a significant difference between these indices was observed at 90 min ($p < 0.05$). The chemotaxis indices for diacetyl under low and high populations at 90 min were 0.73 ± 0.03 and 0.58 ± 0.07 , respectively. The chemotaxis index at 90 min showed a significant negative correlation with the number of animals on the plate ($r = 0.41927$, $p < 0.001$) (Fig. 2D).

Under the condition of a high population, the nema-

Fig. 2. Chemotactic responses to sodium acetate and diacetyl. **(A)** Time course of change in average chemotaxis index for sodium acetate. The value under the condition of a high population (107 ± 14 animals on a plate) was significantly lower than that under the condition of a low population (32 ± 8 animals on a plate) (* $p < 0.05$). **(B)** Correlation between chemotaxis index for sodium acetate at 90 min and number of animals on assay plate. Chemotaxis index showed a significant negative correlation with the number of animals on the plate ($r = 0.57368$, $p < 0.001$). **(C)** Time course of change in average chemotaxis index for diacetyl. The value under the condition of a high population (125 ± 26 animals on a plate) was lower than that under the condition of a low population (37 ± 5 animals on a plate) (* $p < 0.05$). **(D)** Correlation between chemotaxis index for diacetyl at 90 min and number of animals on assay plate. Chemotaxis index showed a significant negative correlation with the number of animals ($r = 0.41927$, $p < 0.001$). Error bars indicate SEM ($N = 10$ assays).

todes that gathered at the attractant location were removed from the attractant location of sodium acetate every 10 min or at 40 min during the assay to confirm the effect of population density at the attractant location (Fig. 3A). When the animals were removed every 10 min or at 40 min, the chemotaxis indices at 90 min were significantly greater than that for the control assay ($p < 0.05$) (Fig. 3B). These results indicate that an increase in population density at an attractant location affects a chemotactic response, suggesting that attraction to chemicals is regulated by some substances produced by nematodes.

Chemotactic response of *daf-22 (m130)* mutants

It is known that *daf-22 (m130)* mutants show a defective secretion of the pheromone but can sense such a pheromone (Golden and Riddle, 1984; 1985). The chemotactic response of *daf-22 (m130)* mutants under the condition of low and high population densities was observed. Under both

conditions of low and high populations, the chemotaxis index for sodium acetate increased rapidly within 30 min and then continued to increase gradually during the assay (Fig. 4A). There was no significant difference in the chemotaxis index between the conditions of low and high populations. The chemotaxis indices for sodium acetate at 90 min under low and high populations were 0.79 ± 0.04 and 0.82 ± 0.04 , respectively. A nonsignificant difference in the chemotaxis index for diacetyl between the conditions of low and high populations was also observed (Fig. 4B). The indices for diacetyl at 90 min under low and high populations were 0.71 ± 0.04 and 0.74 ± 0.04 , respectively.

Effect of pheromone on chemotactic response

The dauer pheromone of *C. elegans* is used as a measure of population density (Golden and Riddle, 1984). From the results of the above experiments, we have speculated that the attraction to chemicals is regulated by an increase

Fig. 3. Effect of population density at attractant location on chemotaxis index for sodium acetate. **(A)** Scheme of experiment. Under the condition of a high population, the animals that gathered at the attractant location were removed from the attractant location every 10 min or at 40 min during the assay. **(B)** Change in chemotaxis index at 90 min among experimental groups. When the nematodes were removed every 10 min or at 40 min, the chemotaxis indices at 90 min were significantly greater than that for the control assay (* $p < 0.05$). In the control assay, the lid of the plate was opened but the animals were not removed from the test location. Note that an increase in population density at an attractant location affects the chemotactic response. Error bars indicate SEM (N=12 assays).

Fig. 4. Time course of change in average chemotaxis index of *daf-22 (m130)* mutants for sodium acetate **(A)** and diacetyl **(B)**. Note that there was no significant difference in the chemotaxis index between the conditions of low and high populations regardless of the type of attractant. Error bars indicate SEM (N≥11 assays).

in pheromone concentration at an attractant location. To confirm this possibility, the effect of a high population density at a particular attractant location on chemotaxis was investigated.

When the nematodes were placed at the attractant location in advance, the chemotactic response of the animals at the original location was determined (Fig. 5A). When *daf-22 (m130)* mutants of a high population density were placed at the attractant location in advance (*daf-22/N2* assay), the chemotaxis index of N2 animals for sodium acetate was almost the same as that of the control assay (*-/Na* assay) in which no animals were placed at the attractant location in advance (left panel in Fig. 5B). However, when N2 animals of a high population density were placed at the

attractant location in advance (N2/N2 assay), the chemotaxis index of the animals for sodium acetate was significantly smaller than those of the control (*-/N2*) and *daf-22/N2* assays ($p < 0.05$) (left panel in Fig. 5B). The chemotaxis indices were 0.66 ± 0.03 in N2/N2, 0.74 ± 0.03 in *-/N2* and 0.74 ± 0.03 in *daf-22/N2* assays.

When the N2 animals of a high population density were placed at the attractant location in advance (N2/*daf-22* assay), the chemotaxis index for sodium acetate of *daf-22 (m130)* mutants was significantly smaller than that of the control (*-/daf-22* assay) in which no animals were placed at the attractant location in advance ($p < 0.05$) (right panel in Fig. 5B). The indices were 0.70 ± 0.03 in N2/*daf-22* and 0.81 ± 0.03 in *-/daf-22* assays.

Fig. 5. Effect of a high population density at attractant location on chemotaxis index. **(A)** Scheme of experiment. Approximately 100 nematodes were placed at the attractant location 60 min before the assay, and then approximately 30 nematodes marked with Indian ink were placed at the original locations (C and D), and their chemotactic responses were observed for 90 min. **(B)** Change in chemotaxis index for sodium acetate at 90 min among experimental groups. **(C)** Change in chemotaxis index for diacetyl at 90 min among experimental groups. N2 animals gathered at the attractant location, which was the same as that in the case of the control assay in which no animals were placed at the attractant location in advance, even when *daf-22* (*m130*) mutants of a high population density were placed at the attractant area in advance (*daf-22*/N2 assay). On the other hand, when N2 animals of a high population density were placed at the attractant location in advance (N2/*daf-22* assay and N2/N2 assay), the chemotaxis index of N2 animals or *daf-22* (*m130*) mutants was significantly smaller than that obtained in the control assay. Error bars indicate SEM (N=16 assays). * $p < 0.05$.

Similar to the response to sodium acetate, when *daf-22* (*m130*) mutants of a high population density were placed at the attractant location in advance (*daf-22*/N2 assay), the chemotaxis index of N2 animals for diacetyl was almost the same as that of the control assay (-/N2 assay) in which no animals were placed at the attractant location in advance (left panel in Fig. 5C). In contrast, when N2 animals of a high population density were placed at the attractant area in advance (N2/N2 assay), the chemotaxis index for diacetyl

was significantly smaller than those of the control (-/N2) and *daf-22*/N2 assays ($p < 0.05$) (left panel in Fig. 5C). The indices were 0.46 ± 0.03 in -/N2, 0.46 ± 0.06 in *daf-22*/N2 and 0.28 ± 0.04 in N2/N2 assays.

When N2 animals of a high population density were placed at the attractant area in advance (N2/*daf-22* assay), the chemotaxis index for diacetyl of *daf-22* (*m130*) mutants was significantly smaller than that of the control assay (-/*daf-22* assay) in which no animals were placed at the attractant

location in advance ($p < 0.05$) (right panel in Fig. 5C). The chemotaxis indices were 0.36 ± 0.04 in N2/daf-22 and 0.54 ± 0.05 in -/daf-22 assays.

The chemotaxis indices for diacetyl in above assays were smaller than those of the previous experiments (Figs. 2C and 4B). In the assays, we spotted diacetyl at an attractant location of an assay plate 1 hr before the start of the assay to ensure a high population density of nematodes at the attractant location in advance, whereas it was spotted shortly before the assay in the previous experiments. Therefore, the difference in the indices was caused by a change in the efficacy of diacetyl at the attractant location.

As described above, *daf-22* (*m130*) mutants cannot

secrete but can sense the pheromone. These results suggest that an increase in pheromone concentration at an attractant location inhibits the attractive response of nematodes.

Response to pheromone produced by N2 animals

In the above experiments, we investigated the effect of a substance produced by N2 animals, *i.e.*, a pheromone, on the chemotaxis, and revealed that the chemotactic response of the nematode was inhibited by the pheromone. The question arose as to whether the pheromone is a repellent or not. To clarify this question, we had to directly investigate the effect of the pheromone on animal's behavior in the absence of an attractant. However, it was difficult for us to purify the

Fig. 6. Effect of pheromone produced by N2 animals on nematode's behavior. **(A)** Scheme of experiment. The plug in the absence of animals or containing approximately 100 *daf-22* (*m130*) mutants placed at location A, and the plug containing approximately 100 N2 animals or *daf-22* (*m130*) mutants was placed at location B. Approximately 30 N2 animals or *daf-22* (*m130*) mutants marked with Indian ink were placed at the midline of the assay plate. The numbers of animals, which moved from the original area, at the test and control areas were determined for 60 min. **(B)** Confirmation of efficacy of pheromone on chemotaxis in this experimental procedure. When N2 animals of a high population density were placed at the attractant area in advance (-:N2+Na assay), the population index of N2 animals for sodium acetate declined in comparison with that for only sodium acetate (-:Na assay). **(C)** Response to pheromone produced by nematodes. The population indices of -:N2, -:daf-22 and daf-22:N2 assays were not significantly different from that of the control assay (-:- assay). Open and closed circles are population indices of individual assay and means, respectively. **(D)** Fraction of animals at the test and control areas. White bars, fraction of animals at the control area; gray bars, fraction of animals at area of *daf-22* (*m130*) mutants of a high population density; black bars, fraction of animals at area of N2 animals of a high population density. Error bars indicate SEM (N ≥ 20 assays). * $p < 0.05$.

pheromone. Therefore, the responses of nematodes to N2 animals and/or *daf-22 (m130)* mutants of a high population density were tested in the absence of an attractant using a different assay plate configuration (Figs. 2B and 6A). This configuration has an advantage for determining the effect of the repellent, and has usually been used for repellent assay (Tajima *et al.*, 2001).

We confirmed that the pheromone reduced the attraction to chemicals by this experimental procedure. When N2 animals of a high population density were placed with sodium acetate in advance at location B in the test area (-:N2+Na assay), the chemotaxis index of N2 animals for sodium acetate was significantly smaller than that for only sodium acetate (-:Na assay) (Fig. 6B). This result indicates that the effect of pheromone produced by N2 animals was reviewed in this measurement.

When N2 animals were placed in the original area, and *daf-22 (m130)* mutants of a high population density were placed at location B in the test area (-:daf-22 assay), there was no significant difference in the fraction of N2 animals between the test and control areas (left panel in Fig. 6D). However, when N2 animals of a high population density were placed at location B in the test area (-:N2 assay), the fraction of animals at the test area was significantly smaller than that at the control area in which no animals were placed in advance ($p < 0.05$) (left panel in Fig. 6D). Moreover, when *daf-22 (m130)* mutants and N2 animals were placed at location A in the control area and at location B in the test area, respectively, (daf-22:N2 assay), the fraction of animals at the test area (side of N2 animals) was smaller than that at the control area (side of *daf-22 (m130)* mutants) ($p < 0.05$) (left panel in Fig. 6D). On the other hand, the population indices of -:N2, -:daf-22 and daf-22:N2 assays were not significantly different from that of the control assay (-:- assay) (Fig. 6C), indicating that an avoidance response of nematodes to the pheromone was not observed in the absence of the attractant. This result suggests that the pheromone does not affect the behavior of the nematodes as a strong repellent.

Similar responses were also observed when *daf-22 (m130)* mutants were placed in the original area. We detected no significant difference in the population index among assay groups of *daf-22 (m130)* mutants (Fig. 6C). On the other hand, when N2 animals of a high population density were placed at location B in the test area (-:N2 assay and daf-22:N2 assay), the fraction of *daf-22 (m130)* mutants at the test area was significantly smaller than that at the control area (right panel in Fig. 6D). These results suggest that the pheromone produced by N2 animals inhibits the entrance of the nematodes to an area of N2 animals of a high population density.

DISCUSSION

In the present study, we investigated the effect of a nematode population on chemotaxis and revealed that the

chemotactic response is affected by the nematode population. The chemotaxis index of N2 animals decreased with an increase in the number of animals on the assay plate regardless of the type of attractant, *i. e.*, water-soluble substrate (sodium acetate) or volatile odorant (diacetyl) (Fig. 2). Nuttly *et al.* (2001) also reported that the time of aversive response to 100% benzaldehyde for the individually tested animals is shorter than that for the group-tested animals. These observations suggest the existence of interactions between individuals during population assays.

Two possible mechanisms underlying the interactions between individual animals are proposed. One is the suppression of chemical stimulation by mechanical stimulation during population assays. Mechanical stimulation, which is received by animals at the original location, may be enhanced with an increase in the number of animals on a plate due to contact with each other, and may compete with chemical stimulation. However, we rule out this possibility. In spite of the condition of a high population density at the original location, when N2 animals were removed from the attractant location, the chemotaxis index showed a significant increase in comparison with that for the control assay in which no nematodes were removed from the attractant location (Fig. 3). Moreover, the chemotaxis response of *daf-22 (m130)* mutants under the conditions of a high population at the original location was almost the same as that under the conditions of a low population (Fig. 4). These results indicate that increase in mechanical stimulation by a high population density at the original location does not affect attraction to chemicals.

Another possible mechanism is interaction with substances such as pheromones. We propose the existence of a chemotaxis regulation pheromone (CR-pheromone). *daf-22 (m130)* mutants do not form dauers if crowded and starved, and a pheromone extract prepared from these mutants is not capable of inducing dauer formation in N2 animals (Golden and Riddle, 1985). However, these mutants are capable of forming dauers in response to exogenously supplied pheromones (Ailion and Thomas, 2000). This suggests that *daf-22 (m130)* mutants are defective in secretion of pheromones including the dauer pheromone but respond normally to the pheromones. On the other hand, wild type N2 animals produce the dauer pheromone and use it to measure population density (Golden and Riddle, 1984). In the present study, when N2 animals of a high population density were placed at the attractant location in advance, the chemotaxis indices of both N2 animals and *daf-22 (m130)* mutants were significantly lower than those in the case of the control in which no animals were placed at the attractant location in advance (Fig. 5). In contrast, both N2 animals and *daf-22 (m130)* mutants could gather at the attractant location, which was the same as in the control assay in which no animals were placed at the attractant location in advance, even when *daf-22 (m130)* mutants of a high population density were placed at the attractant location in advance (Figs. 4 and 5). Because of the defective

CR-pheromone secretion by *daf-22 (m130)* mutants, the concentration of pheromone at the attractant location did not increase. Therefore, the nematodes could gather at the attractant location. The CR-pheromone is one of the substances which is produced by N2 animals but not *daf-22 (m130)* mutants, although it is not clear that the CR-pheromone is the same substance as the dauer pheromone.

In the absence of an attractant, we observed a decline in the fraction of animals at the area of N2 animals of a high population density, although the nematodes were not influenced by *daf-22 (m130)* mutants of a high population density (Fig. 6D). On the other hand, there was no significant difference in population indices among assays for N2 animals, *daf-22 (m130)* mutants and the control (Fig. 6C). These results imply two opposite possibilities. The first possibility is that the CR-pheromone produced by N2 animals is a repellent, but the effect of the CR-pheromone as a repellent was not detected in the experiment because of a larger test area in comparison with the attractant location of chemotaxis assay. However, in this assay for the pheromone, we confirmed that the population index of N2 animals for sodium acetate, which was calculated using the number of animals in the test and control areas, was reduced by CR-pheromone (Fig. 6B). This indicates that the CR-pheromone has an effect on an animal's behavior in the test area of this assay. The second possibility is that the CR-pheromone is not a repellent, but inhibits the entrance of the nematodes to a high-pheromone-concentration area. In this experiment, we revealed that the CR-pheromone was not a strong repellent. However, we could not conclude whether the CR-pheromone is a weak repellent or not. Further experiments, such as pheromone purification or *daf-22* cloning, are required to clarify the mechanisms of the pheromonal regulation of chemotaxis.

We revealed that attraction to chemicals is inhibited by an increase in the CR-pheromone concentration at an attractant location due to an increase in population density. Why does *C. elegans* need the mechanism of population density regulation at an attractant location? *C. elegans* can chemotax toward the peak of a gradient of a number of small molecules, such as Na⁺, K⁺, Cl⁻, cAMP, lysine and cysteine, which are produced by food bacteria (Ward, 1973; Dusenbery, 1974). The signal of these substances indicates the existence of food for the nematode, and the chemotactic behavior of *C. elegans* may arise as a result of food detection. When many animals gather at a food source, the volume of food that each individual receives is smaller, and the decrease in the amount of food is faster than that when only a few animals gather at food location. Pheromones are produced by one animal to communicate with each other and initiate a range of physiological responses. For individual nematodes to survive, the mechanism of population density regulation at an attractant (food) location by pheromone release may have developed during their evolution.

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