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Migration of *Caenorhabditis elegans* (Nematoda : Rhabditidae) larvae towards bacteria and the nature of the bacterial stimulus

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Summary — Chemotaxis by *Caenorhabditis elegans* larvae to several species of bacteria was studied on agar in specially designed Petri plates. All the bacteria affected the pattern of nematode migration and the degree of this alteration depended on the species of bacteria. *Acinetobacter calcoaceticus* var. *anitratus, Enterobacter amnigenus, E. cloacae, Pseudomonas maltophilia* and *Serratia liquefaciens* were more attractive than *Escherichia coli* and *Pseudomonas fluorescens* biovar *reactans. Bacillus cereus, B. thuringiensis* and *Bacillus* sp. showed least influence on nematode migration. Younger bacterial colonies (24-48 h old) were more attractive than the older ones (96-192 h old). When killed *in-situ* by chloroform fumes, bacterial colonies remained attractive to the nematode. Methods were developed to study the nature of the bacterial stimulus and the involvement of both diffusible and/or volatile attractants was found.

Résumé — ***Migration des larves de* Caenorhabditis elegans *(Nematoda : Rhabditidae) vers les bactéries, et nature du stimulus bactérien*** — Le chimiotactisme de *Caenorhabditis elegans* vis-à-vis de plusieurs espèces de bactéries a été étudié dans des boîtes de Petri spécialement conçues. Toutes les bactéries testées ont un effet sur le mode de migration des nématodes et le degré de modification dépend des espèces de bactéries. *Acinetobacter calcoaceticus* var. *anitratus, Enterobacter amnigenus, E. cloacae, Pseudomonas maltophilia* et *Serratia liquefaciens* sont plus attractifs qu'*Escherichia coli* et *Pseudomonas fluorescens* biovar *reactans. Bacillus cereus, B. thuringiensis* and *Bacillus* sp. n'ont qu'une faible influence sur la migration. Les colonies bactériennes jeunes (24-48 h) sont plus attractives que les colonies âgées (96-192 h). Lorsqu'elles sont tuées par des vapeurs de chloroforme, les colonies bactériennes restent attractives pour le nématode. Des méthodes sont décrites pour étudier la nature du stimulus bactérien, et la mise en jeu d'attractifs volatils ou diffusant dans la gélose a été trouvée.

Key-words : *Caenorhabditis*, migration, bacteria.

Nematodes respond to a variety of external stimuli, e.g. mechanical, light, temperature, electrical and chemical (Croll, 1970; Dusenbery, 1980; Coomans & De Grisse, 1981). Chemotaxis, mediated by movement along chemical gradients, plays an important role in food-finding, mate-finding and other aspects of nematode interactions (Green, 1980; Zuckerman & Jansson, 1984). However, none of the natural chemicals mediating these responses have been identified.

Chemotaxis by the microbivorous nematode *Caenorhabditis elegans* to a variety of known chemicals is well established (Ward, 1973; Dusenbery, 1980, 1983). It is attracted to cyclic AMP, Na^+, Cl^-, OH^-, pyridine, O_2, CO_2 (in borate buffer, pH 8.8) and repelled by CO_2 (in phosphate buffer, pH 6.0), D-tryptophan, H^+ and high osmotic pressure (Dusenbery, 1983). Chemotactic signals are received at the amphids and the inner labial sensillae which are located in the cephalic region of nematodes (Coomans & De Grisse, 1981; Dusenbery, 1983). Although the anatomy of cephalic sense organs of *C. elegans* has been studied in detail (Ward *et al.*, 1975; Ware *et al.*, 1975; Wright, 1983) there is no direct evidence on the manner in which reception of chemotactic signal leads to an orientated response.

The attraction of bacterial-feeding nematodes to their natural food source has been studied by several investigators (Andrew & Nicholas, 1976; Hosono, 1978; Jansson & Nordbring-Hertz, 1983). Andrew and Nicholas (1976) found that *C. elegans* was attracted to three bacterial species on which it could feed and was repelled by one toxic species indicating the importance of attraction to a suitable food. However, the nature of the bacterial stimulus mediating these interactions was not known. The present paper reports on the effects of several species of bacteria on the migration of *C. elegans* on agar plates and also presents evidence for both the volatile and diffusible nature of the bacterial stimulus.

Material and methods

NEMATODE CULTURE

C. elegans was isolated from a sample of compost collected from a mushroom farm near Taunton, Somerset, U.K. The nematode was cultured with associated bacteria at 22 °C on 3 % (w/v) nutrient agar (Oxoid Ltd.) in Petri plates. In all the experiments, third and fourth stage larvae were selected from 8-10 day old cultures and surface sterilized with 0.1 % " Thimerosal " (w/v sodium ethyl mercurithiosalicylate, Sigma Ltd.) following Grewal (1990). The sterile larvae were left in sterile distilled

water in cavity blocks for 24 h at 22 °C and then used for attraction studies.

Bacterial cultures

Ten species of bacteria out of the thirteen tested in this study were isolated from the Taunton strain of *C. elegans* (Grewal, 1990). They included; *Acinetobacter calcoaceticus* var. *anitratus*, *Bacillus cereus*, *Bacillus* sp., *Enterobacter amnigenus*, *E. cloacae*, *Pseudomonas aeruginosa*, *P. fluorescens* biovar *reactans*, *P. maltophilia*, *Pseudomonas* sp. and *Serratia liquefaciens*. Reference strains of *Bacillus thuringiensis* (HD 1), *Escherichia coli* (ATCC 9001) and *Pseudomonas tolaasii* (Pt 51) were obtained from the HRI culture collection. All the bacteria except *P. tolaasii* were maintained on 3 % nutrient agar plates, stored at 4 °C and were subcultured at monthly intervals. *P. tolaasii* was maintained on King's B medium (King *et al.*, 1954).

Quantification of nematode response

Quadrant plates were prepared according to Andrew and Nicholas (1976) with concentric rings marked at an interval of one cm on the bottom of each Petri plate (8 cm diam., Fig. 1). Nutrient agar was poured into the plates and a loop-full of bacteria, from 24 h old cultures grown in nutrient broth in shaker flasks at 25 °C, was streaked along a quarter of the periphery (Fig. 1). The plates were then incubated at 25 °C for 24 h. Nematodes (60 ± 1.8) contained in 30 µl of sterile distilled water were placed in the centre of each plate, the drops were then allowed to evaporate for 5 min on a sterile-air laminar flow cabinet. The number of nematodes which moved out of the central ring towards the bacterial streak (test side) and the bacteria-free side (control side) were recorded periodically. The mean distance travelled by nematodes towards bacteria after each time interval in each plate (replicate) was calculated using the equation;

$$\bar{X} + [1\,t_1 + 2\,t_2 + 3\,t_3 - (1\,c_1 + 2\,c_2 + 3\,c_3)] \cdot T^{-1}$$

where the numbers of nematodes in the respective arcs of the test and in the control quadrant are represented by t_1, t_2, t_3 and c_1, c_2, c_3, respectively. The numerals *1*, *2* and *3* represent the distance in centimeters of the respective arcs from the centre to the periphery and *T* is total number of nematodes added. All experiments were conducted at a room temperature of 20-21 °C and the data were subjected to analysis of variance.

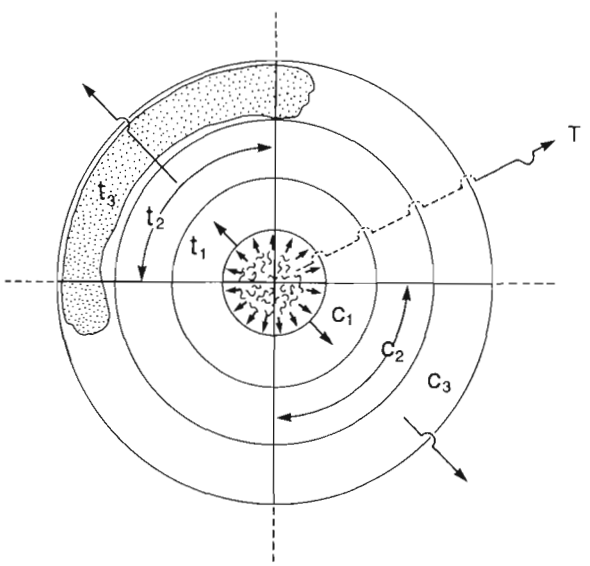


Fig. 1. A quadrant plate used to quantify the nematode response to the test material (dotted area). T = total number of nematodes added; t_1, t_2, t_3 are the respective arcs in the test quadrant; and c_1, c_2, c_3 are the respective arcs in the control quadrant. Attraction index is defined as the mean distance travelled by nematodes at any time from the central arc towards the periphery (see text for details).

Relative attractiveness of bacterial species

Using the above technique, the effects of 24 h-old colonies of thirteen species of bacteria, growing on nutrient agar, on the migratory behaviour of *C. elegans* were studied. Bacteria were randomly divided into four groups; one group contained four species and the others used three species each. Three replicate plates were prepared for each species of bacteria and three bacteria-free control plates containing streaks of nutrient broth were run simultaneously. The response of nematodes to bacteria were assessed periodically and the data analysed as above.

Response of nematodes to different aged bacterial streaks

Streaks of different ages of culture (24, 48, 96 and 192 h old) of two attractive bacteria; *A calcoaceticus* var. *anitratus* and *S. liquefaciens*, grown at 25 °C, were tested for their effects on nematode attraction. One species of bacteria was studied at a time with three replicates of each treatment. The data on the mean distance travelled by nematodes in each plate was recorded periodically and analysed as above.

Response of nematodes to dead bacteria

Four species of attractive bacteria including, *A calcoaceticus* var. *anitratus*, *E. amnigenus*, *P. maltophilia*, and *S. liquefaciens* were streak-inoculated, individually, around a quarter of the periphery of quadrant plates. Three plates were used for each species. The plates were incubated at 25 °C for 24 h. The bacterial cultures were then killed *in situ* by exposing the plates to drops of chloroform (50 µl each) for 30 min in a fume cupboard. To test the effectiveness of chloroform treatment, a loop-full of bacteria from each lawn was inoculated onto fresh nutrient agar plates and examined for next three days for any growth of the bacteria at 25 °C. The

response of *C. elegans* to the dead bacterial streaks was studied (immediately after killing) as described earlier.

NATURE OF ATTRACTANTS

Volatile attractants

Non-vented quadrant plates were divided into two unequal portions by fitting aseptic impermeable plastic barriers to the sides and bottom of the plate with " UHU " glue (Beecham, Brentford, UK) before pouring the agar (Fig. 2). The level of the agar was kept a little below the partition wall. Bacteria were streaked in the partitioned area and incubated at 25 °C for 24 h. After the introduction of nematodes in the centre, the quadrant plates were sealed with Sellotape and responses of nematodes to all the thirteen species of bacteria were assessed periodically as described earlier.

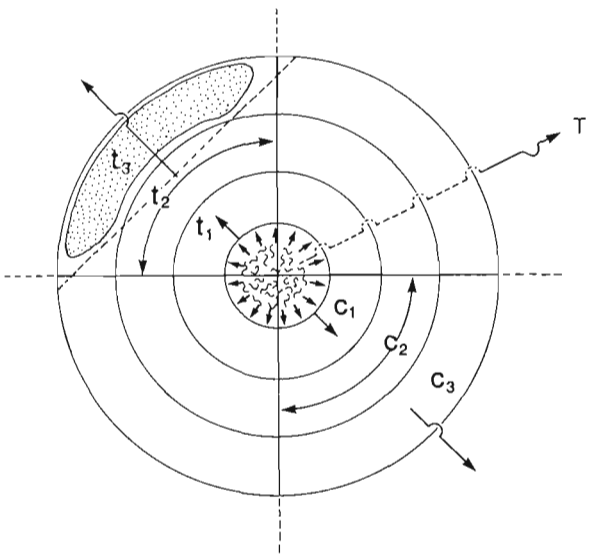


Fig. 2. Modified quadrant plate to evaluate the volatile nature of the bacterial stimulus. Dotted line in the test quadrant shows the place of plastic barrier. For other details refer to Fig. 1.

Diffusible attractants

For detection of diffusible attractants, bacteria were grown on nutrient agar plates in straight lines at an interval of one cm. After 24 h incubation at 25 °C, the inter-streak portions were taken out with a sterile scalpel and placed on half the periphery (test side) of prepoured nutrient agar quadrant plates. In the other half of the periphery (control side), were placed similarly sized nutrient agar blocks obtained from the bacteria-free plates. Control plates received agar blocks from the bacteria-free plates in both halves of the periphery. Relative migration of nematodes towards both the test and control sides were estimated as described above.

Diffusible-volatile attractants

Agar blocks containing the bacterial diffusates were prepared in similar conditions of incubation. These agar-blocks were then placed on thin pieces of microscope slide cover-glass in half the periphery of prepoured nutrient agar plates. The other half of these plates (i.e. the control sides) and also the separate control plates received agar-blocks from bacteria-free plates. Four species of attractive bacteria (that showed the production of diffusible attractive substance) were studied simultaneously with three replications for each species. Relative migration of nematodes towards the test or control sides was assessed periodically as described above.

Results

RELATIVE ATTRACTIVENESS OF BACTERIAL SPECIES

All the species of bacteria studied altered the normal random movement of the test nematode to a more directed and precise orientation towards bacterial colonies (Fig. 3). The degree of alteration in the pattern of nematode migration varied with the bacterial species. For instance, *A. calcoaceticus* var. *anitratus, E. amnigenus, E. cloacae, P. maltophilia* and *S. liquefaciens* elicited a significant ($P < 0.05$) and rapid response of the nematodes when compared with any other bacteria.

Bacillus species including *B. cereus, B. thuringiensis* and *Bacillus* sp. showed the least effects on the nematode migration and attracted fewest nematodes. Other bacteria such as *E. coli, P. aeruginosa* and *P. fluorescens* biovar *reactans, P. tolaasii* and *Pseudomonas* sp. produced intermediate effects.

RESPONSE OF NEMATODES TO DIFFERENT AGED BACTERIAL STREAKS

Differential response was observed when the nematodes were exposed to different aged bacterial streaks (24, 48, 96, 192 h old) of two attractive bacteria (Fig. 4). The 24 h old streaks of *A. calcoaceticus* var. *anitratus* were most attractive whereas 48 h old streaks of *S. liquefaciens* elicited significantly vigorous response. Furthermore, the streaks of *A. calcoaceticus* var. *anitratus* remained attractive even after 192 h incubation whereas the same aged lawns of *S. liquefaciens* were not attractive.

RESPONSE OF NEMATODES TO DEAD BACTERIA

Nematodes were attracted towards dead streaks of all the bacteria tested (Fig. 5). Three bacteria namely *A. calcoaceticus* var. *anitratus, E. cloacae,* and *S. liquefaciens* were significantly ($P < 0.05$) more attractive than *P. maltophilia.*

NATURE OF ATTRACTANTS

Volatile attractants

Fig. 6 shows the effects of bacteria on the orientation

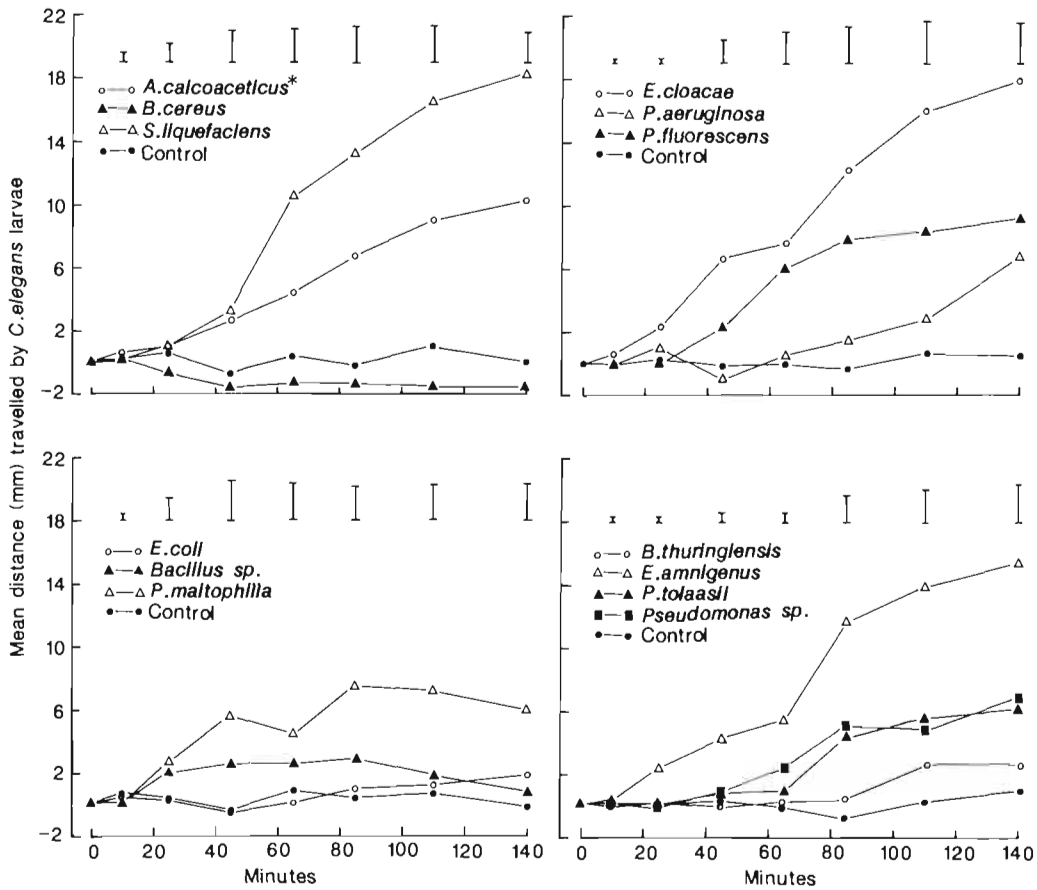


Fig. 3. Effects of intact bacterial streaks of different species on the migration of *C. elegans*. Data are mean distances (mm) travelled by the third stage larvae towards or away from the bacterial streak. Bars represent LSD ($p < 0.05$). **A. calcoaceticus* var. *anitratus*.

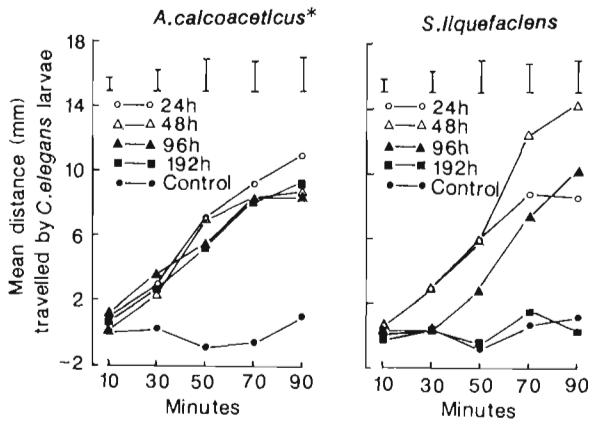


Fig. 4. Effects of age of bacterial streaks on the migratory behaviour of *C. elegans*. Data are mean distances (mm) travelled by the third stage larvae towards or away from the bacterial streaks. Bars represent LSD ($p < 0.05$). **A. calcoaceticus* var. *anitratus*.

of *C. elegans* on partitioned quadrant plates when the bacterial streak was separated from the rest of the plate to avoid any diffusion through the agar. All the species of bacteria except *Bacillus* sp. and *P. fluorescens* biovar *reactans* produced volatile attractants which significantly ($P < 0.05$) affected the nematode migration. *B. thuringiensis* was more attractive to the nematodes on partitioned plates (Fig. 6) than on the plates where diffusion through the agar was unimpeded (Fig. 3).

Diffusible attractants

Agar-blocks containing the bacterial diffusates elicited differential response of the nematodes (Fig. 7). Diffusates of *A. calcoaceticus* var. *anitratus, E. amnigenus, E. cloacae, P. aeruginosa, P. fluorescens* biovar *reactans, P. maltophilia, P. tolaasii, Pseudomonas* sp. and *S. liquefaciens* had significant ($P < 0.05$) attractive effects on the nematodes whereas those of *B. cereus, B. thuringiensis, Bacillus* sp. and *E. coli* did not elicit any significant ($P < 0.05$) response.

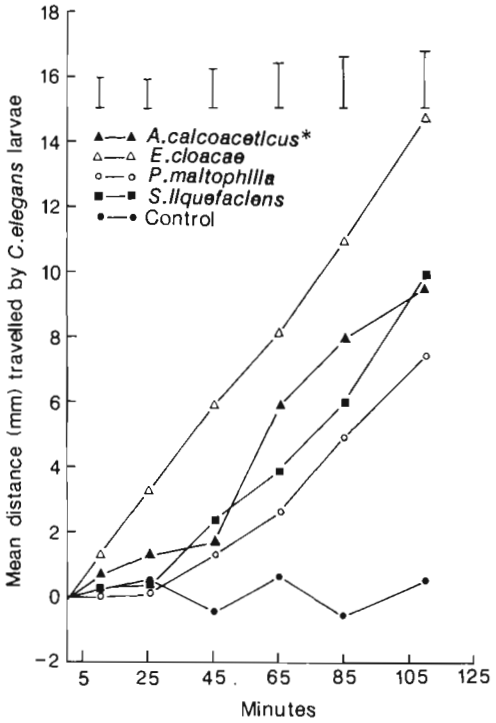
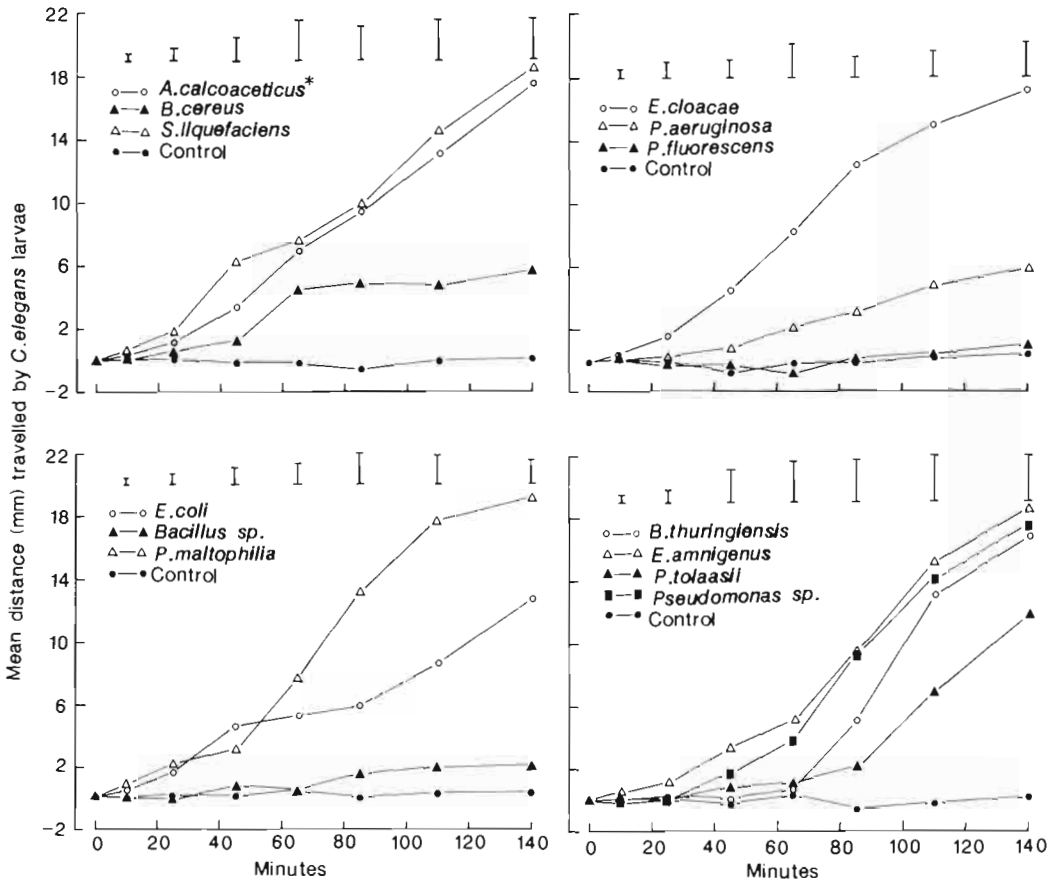


Fig. 5. Effects of dead bacteria (killed *in-situ*) on the migratory behaviour of *C. elegans*. Data are mean distances (mm) travelled by the third stage larvae towards and away from the bacterial streak. Bars represent LSD ($p < 0.05$). **A. calcoaceticus* var. *anitratus*.

Fig. 6. Effects of different bacteria on the migratory behaviour of *C. elegans* when the diffusion through the agar was impeded. Data are mean distances (mm) travelled by the third stage larvae towards and away from the bacterial streak. Bars represent LSD ($p < 0.05$). **A. calcoaceticus* var. *anitratus*.



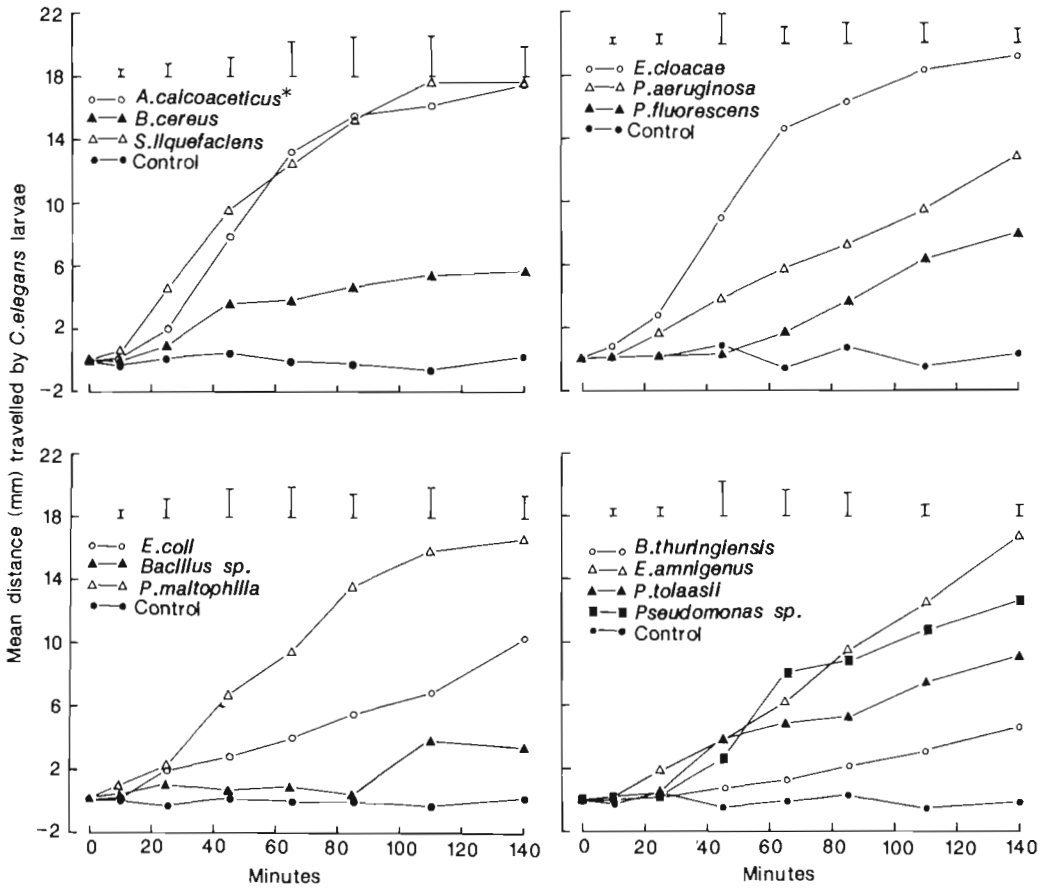


Fig. 7. Effects of diffusates (contained in agar blocks) of different bacterial species on the migratory behaviour of *C. elegans*. Data are mean distances (mm) travelled by the third stage larvae towards and away from the test blocks. Bars represent LSD ($p < 0.05$). **A. calcoaceticus* var. *anitratus*.

Diffusible-volatile attractants

Fig. 8 shows that the agar blocks containing bacterial diffusates emitted volatile substances that attracted nematodes. The agar blocks containing diffusates of the bacteria, *E. amnigenus*, *E. cloacae* and *S. liquefaciens* attracted significantly ($P < 0.05$) more nematodes than *A. calcoaceticus* var. *anitratus*.

Discussion

The present study has shown that the presence of bacteria affected the pattern of *C. elegans* migration on agar plates and that the degree of alteration depended on the species of bacteria. Similar observations have been reported in the past on *C. elegans* (Andrew & Nicholas, 1976) and on *Neoaplectana carpocapsae* (Pye & Burman, 1981). Andrew and Nicholas (1976) reported that *Escherichia coli*, *Pseudomonas fluorescens* and *P. aeruginosa* were the most attractive bacteria for *C. elegans*. *Bacillus mycoides* and *B. subtilis* were comparatively less attractive and *B. megatherium* was a repellent. The present results expand this list and had shown that some of the bacteria e.g. *A. calcoaceticus* var. *anitratus*, *E. amnigenus*, *E. cloacae*, *P. maltophilia* and *S. liquefaciens* were even more attractive than *E. coli* and *P. fluorescens* biovar *reactans*. The group of bacteria including, *E. coli*, *P. aeruginosa*, *P. fluorescens* biovar *reactans*, *P. tolaasii* and *Pseudomonas* sp. elicited an intermediate response while *B. cereus*, *B. thuringiensis* and *Bacillus* sp. were the least attractive. No bacteria repelled *C. elegans*.

This species-specific response of *C. elegans* to various bacteria may be due to different types and/or concentrations of attractants produced by bacteria. Apart from inherent differences between bacteria, the growth conditions also affect the quality of bacterial cells (Schiemer, 1982). The choice of static populations of bacteria rather than replenishing cultures and also the use of a similar nutrient medium for all the species of

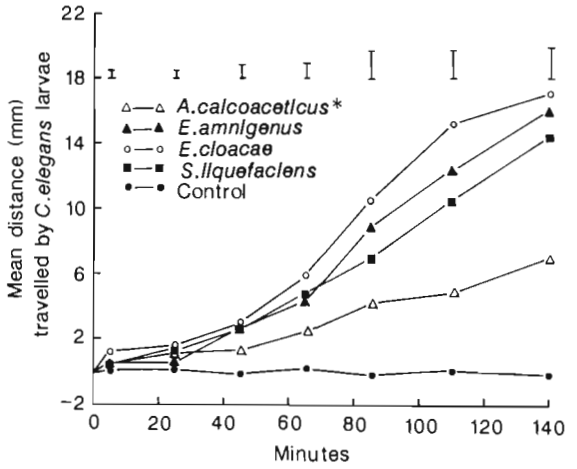


Fig. 8. Effects of diffusates (contained in agar blocks) of different bacteria on the migratory behaviour of *C. elegans* when the further diffusion through the agar was impeded. Data are mean distances (mm) travelled by the third stage larvae towards and away from the test blocks. Bars represent LSD ($p < 0.05$). * *A. calcoaceticus* var. *anitratus*.

bacteria may have directly affected both the quality and quantity of bacterial populations and perhaps also the production of attractants.

The ability of bacteria to attract nematodes is probably related to their growth conditions : 24 h old colonies of *A. calcoaceticus* var. *anitratus* were more attractive than the older colonies. In *S. liquefaciens* the 48 h old colonies were more attractive than 24 h old ones which may be due to a higher optimum growth temperature for this bacterium (28-35 °C : Grimont and Grimont, 1984). This indicates that the attractants are produced in abundance only when the bacteria are in active growth phase. The differential response of *C. elegans* to the old aged cultures of *A. calcoaceticus* var. *anitratus* and *S. liquefaciens* (i.e. 192 h old cultures) suggested the production of different types and/or nature of attractants by the two bacteria.

Andrew and Nicholas (1976) observed that the smears of autoclaved bacteria were not attractive. Therefore, the attractiveness of bacterial streaks that were killed *in situ* by fumes of chloroform may be due to the prior establishment of gradients of attractants in the agar. This view was supported when the agar blocks containing the bacterial diffusates attracted the nematodes.

There is evidence of chemical attraction of nematodes to bacteria although the identity of the chemicals involved is uncertain. Ward (1973) reported that *C. elegans* was attracted to cyclic AMP, which is released by bacteria; and to other unidentified substances in the media where bacteria have grown. Andrew and Nicholas (1976) reported that the attractive bacteria produced an alkaline environment in their vicinity. Therefore, it is also possible that the attraction is based simply on the pH gradient as *C. elegans* is attracted to high pH (Ward, 1973).

Another interesting aspect of this study was the observation that the agar-blocks containing bacterial diffusates emitted volatile attractive substance(s). On the partitioned-quadrant plates, eleven species of bacteria (out of the 13 studied) produced volatiles that affected the nematode migration. Furthermore, the pattern of *C. elegans* response to the volatiles was almost comparable to that when an unimpeded diffusion through the agar occurred. These results suggest that the volatile substances may be the main component(s) of the attractants produced by bacteria. In nature, the volatile attractants may be more important than the diffusible substances in affecting the nematode migration significantly because the diffusion through the substrate is generally impeded.

B. thuringiensis was more attractive when diffusion through the agar was not allowed. This suggests that the bacterial products that diffuse into the agar probably impart selectivity to the nematode's response. *B. thuringiensis* is known to produce exotoxins (Ignoffo & Dropkin, 1977; Sebesta *et al.*, 1981; Faust & Bulla, 1982) that might have affected the migration of nematodes when diffusion through the substrate was unimpeded.

Because of its sufficiently high concentrations in nature, carbon dioxide may be a possible attractant. Klingler (1965) reported that plant parasitic nematodes are attracted to the roots of plants which produce CO_2. Gaugler *et al.* (1980) demonstrated that the infective stage juveniles of *N. carpocapsae* migrated up the CO_2 gradient. Furthermore, the response of nematodes to CO_2 depends upon its concentration in the medium (Balan & Gerber, 1972) and of one of its hydrated forms on the type of other ions present (Dusenbery, 1974). Therefore, even if all species of bacteria produce CO_2, the above complexity of interactions explain the differential response of *C. elegans* to different bacteria.

Conflicting reports exist about the possibility of ammonia as a nematode attractant. Katznelson and Henderson (1963) found that some soil nematodes, though not *Caenorhabditis briggsae*, were attracted to ammonium ions whereas Ward (1973) demonstrated that *C. elegans* was neither attracted nor repelled by ammonium ions. Andrew and Nicholas (1976) found that the more attractive bacteria released ammonium ions into peptone-water solution in which they were grown and also produced alkaline gradient in the agar cultures. Schmidt and All (1979) demonstrated that the " dauer " larvae of *N. carpocapsae* are attracted towards various constituents of insect feces including ammonia. These authors observed that the nematodes migrated onto the top of ammonia treated filter paper squares whereas with the other attractants such as allantoin,

arginine and adenine they aggregated in a dense mass directly beneath the square. This probably explains the effects of volatile nature of ammonia on nematode behaviour.

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