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Chemotaxis of *Bacillus subtilis* to Fungal Exudates and to the Individual Amino Acid Components in Fungal Exudates

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ABSTRACT. *Penicillium decumbens* produced large quantities of free amino acids, peptides and proteins into the culture medium. Chemotactic response of *Bacillus subtilis* to the fungus filtrate and some amino acids was examined. Three times as many bacteria were attracted by the culture filtrate than by the original culture medium. Numbers of bacteria attracted by optimum concentration of leucine, serine or alanine were in the range of $8.2 - 9.9 \times 10^4$ compared to 1.6×10^3 bacteria attracted by the control (chemotaxis medium). The fungus filtrate and the amino acids were still good attractants at high hydrogen ion concentrations. It is postulated that if fungi can exude similar substances in soil, motile bacteria like *B. subtilis* could be attracted to them, provided that a continuous water film exists.

Siala *et al.* (1974) investigated the distribution of *B. subtilis* in Freshfiled pine-forest soil. They showed that *B. subtilis* was present mainly in the vegetative form in the acid horizon and as spores in the alkaline horizon. The association between *B. subtilis* and fungi resulting in the growth of the bacteria in the forest acid soils (Siala and Gray 1974) provides an explanation for the distribution of *B. subtilis* in the forest soil horizons. Whether the bacteria use nutrients excreted by living hyphae or cause the death of the fungus and live on the products of lysis is not known? The possibility that fungi alter the pH of the soil by ammonification and create conditions more favourable for the growth of the bacteria is not ruled out (Siala and Gray 1974). However, the work on the ability of bacteria, including *B. subtilis,* to move in natural and artificial soils and along living and dead fungal hyphae (Wong and Griffin 1976a, b), as well as the intensive work carried out during the last 15 years on chemotactic response and attraction of motile bacteria, even to small concentrations of a wide range of substances, suggest that the observed concentration of bacteria on or near fungal hyphae in soil may be the result of chemotactic movement.

In the present study, experiments were designed to investigate the attraction of *B. subtilis* to fungal exudates and to some of the individual amino acids found in the fungal exudates.

Material and Methods

Organisms

Bacillus subtilis (strain CN2745) was used in this study. It does not use ammonia as a nitrogen source, but can grow when given proline, leucine, serine and asparagine. This amino acid mixture is not minimal. Serine appears to be an absolute requirement in the above mixture and by omitting it from the medium, growth of the organism can be prevented, allowing unambiguous interpretation of the chemotaxis experiments. Increase in bacterial numbers inside the capillary tube (see later) can only be accounted for by movement of organisms into the tube.

The fungus *Penicillium decumbens* Thom (Liverpool, Botany department collection F 27/7), a dominant species in Freshfield pine-forest soils where *B. subtilis* was observed in association with fungal hyphae, was chosen for the present study.

Medium for Growth of B. subtilis

Motility was excellent in the presence of 0.01M phosphate buffer (pH 7.0), 10^{-2} or 10^{-3}M $MgSO_4$; 10^{-4}M EDTA and 0.2 g^{-1} glucose. More than 90% of the bacteria were motile for 2 hours, after which motility gradually decreased. Omission of any of the components of the wash medium abolished motility. Organic and amino acids incorporated in the wash medium as energy sources did not stimulate motility. Of the chemicals tested glucose was the only one that served as an excellent energy source.

Growth of P. decumbens in Culture Media

P. decumbens was grown in shake culture in 250 cm^3 flasks containing 30 cm^3 chemically defined medium of the following composition: $NaNO_3$, 2.0 g; K_2HPO_4, 1 g; KH_2PO_4, 0.85 g; $MgCl_2$, 0.5 g; K_2SO_4, 0.4 g; and glucose 30 g in 1 dm^3 distilled water. Glucose was sterilized separately by filtration and added aseptically to the medium. The falsks were inoculated with fungal spores and incubated at 20°C. Another set of flasks containing Czapek Dox medium was prepared, inoculated, and incubated on a shaker at 25°C. After 7 days incubation period the culture media were filtered to remove mycelium and spores. The filtrate was analysed for amino acids and sugars. An aliquot of the filtrate from the glucose salts medium was tested for the ability to attract *B.subtilis* by the capillary method.

Separation of Amino Acids and Sugars from Culture Filtrates

The amino acids and sugars were separated from the culture filtrates by passing the filtrates through suitable ion exchange resins. A cation exchanger (Amberlite resin IR-120(H^+)), and an anion exchanger (Amberlite resin IR-45(OH^-) analytical grade, BDH Chemicals) were prepared according to the method described by Strong (1957). The fungal filtrate was passed first slowly through the cation exchange column. The amino acids together with the negatively charged salt ions were removed by the column, while the sugars and the positively charged salt ions passed through unaffected. The amino acids were eluted from the cation exchange column with 4M ammonia solution and concentrated in a rotary evaporator under partial vacuum for qualitative and quantitative analysis. The analysis was carried out using an amino acid analyser (Joel, GAH, with digital output). Samples from the concentrated amino acid solutions were hydrolysed with 6M HCl in sealed tubes under vacuum for 24 h at 110°C. The hydrolysed samples were evaporated to dryness in a rotary evaporator at 60°C under partial vacuum and dissolved in citrate buffer (pH 2.2) and diluted with the buffer to the required volume. Hydrolysed and unhydrolysed samples were run on the amino acid analyser.

The sugar solutions were then passed through an anion exchange column for further purification, until the pH of the solution approached 6.0. The rest of the ions were removed by the resins and the deionized sugar solution concentrated and the sugars separated by paper chromatography.

Chemotaxis Assay

The method used was that described by Mesibov and Adler (1972) and Adler (1973). The bacteria were grown as described earlier, in tryptone glucose salt medium, harvested, washed and suspended in chemotaxis medium to an $O.D^{*}_{600}$ of 0.08 which is equivalent to 6×10^7 cells cm^3. The chemical composition of the chemotaxis medium is the same as the wash medium described above. Capillary tubes containing the attractant (amino acid in chemataxis medium) were made from 1 μl disposable pipettes (Microcaps, disposable pipettes, Drummond Scientific Company, U.S.A.). The capillaries were inserted in about 0.2 cm^3 bacterial suspension in the chamber and the set-up (Fig. 1) was incubated at 30°C for 20 minutes. The contents of the capillaries were then transferred to 9 cm^3 of 0.2% peptone broth. The peptone was diluted as required and 0.1 cm^3 was plated (1% tryptone, 0.5% sodium chloride). After overnight incubation at 37°C the colonies were counted. As a control capillaries containing chemotaxis medium were also included. The results reported in this paper are based on measurements with three capillariers per single assay.

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* Abbreviation $O.D_{600}$ = optical density measured at 600 nm.

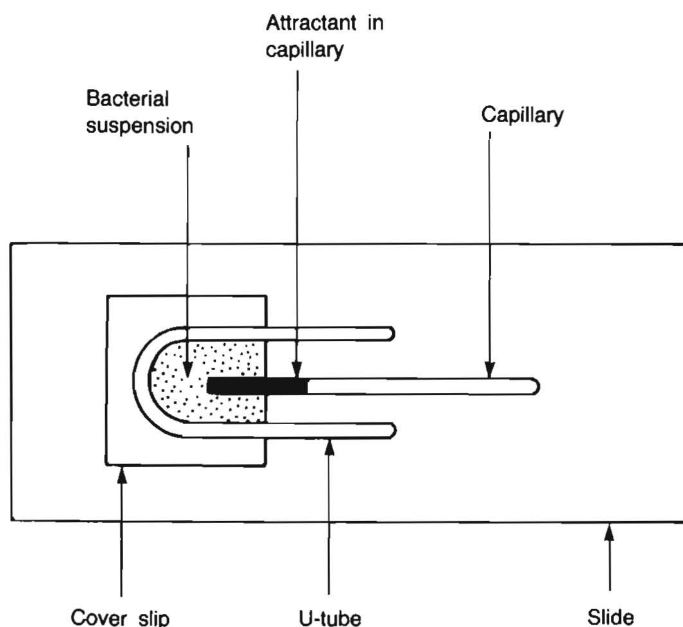

Fig. 1. Apparatus used in the chemotaxis experiment.

Attraction of *B. subtilis* to culture filtrate was also assayed by the capillary method. *P. decumbens* was grown in shake culture in 250 cm^3 flasks containing the glucose salts medium (pH 6.8) mentioned above and incubated for 7 days at 25°C and the medium harvested by filtration. The colour of the medium became slightly brown and the pH dropped from 6.8 to 4.0. An aliquot of the filtrate was adjusted to pH 6.8 by addition of KOH and tested also for the ability to attract *B. subtilis*.

For attraction of *B. subtilis* to leucine and serine at different pH values, solutions of leucine (10^{-3}M) and serine (10^{-4}M) were made up in citrate phosphate buffer at pH 3.0, 5.0 and 7.0. The solutions were placed in the capillary tubes which were subsequently immersed in a bacterial suspension at pH 7.0, the pH optimum for motility.

Results

Effect of Time on Chemotaxis

In order to choose the optimum incubation period for chemotaxis, attraction of bacteria to 10^{-3}M leucine was followed after 10, 20, 30 and 45 minutes. The

highest numbers of bacteria were found in the capillaries after 20 minutes (Fig. 2). The number remained almost stationary for another 10 minutes and then there was a slight decrease in bacterial numbers after a further 15 minutes incubation. In all further experiments an incubation time of 20 minutes was used.

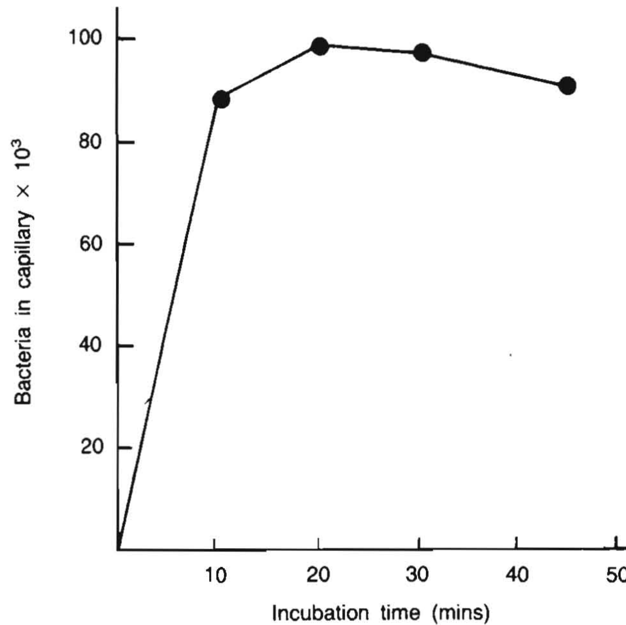

Fig. 2. Effect of time on chemotaxis.

Attraction of B. subtilis to Culture Filtrate

It is clear from Table 1 that three times as many *B. subtilis* cells were attracted by the fungal filtrate than by the original culture medium. There was very little difference between the numbers of bacteria attracted to the culture filtrate at pH 4.0 and 6.8.

Table 1. Attraction of *B. subtilis* to the original culture medium and to the culture filtrate.

Medium	pH	Number of bacteria attracted in capillary
Culture medium	6.8	17,000
Culture filtrate	6.8	44,000
Culture filtrate	4.0	45,000

Analysis of Amino Acids and Sugars

Qualitative and quantitative estimation of amino acids was carried out using an amino acid analyser. The results (Table 2) show that *P. decumbens* was able to excrete aspartic acid, serine, glycine, alanine and peptides into the Czapek Dox culture medium. After hydrolysis, threonine and glutamic acid appeared in the acid hydrolysate together with the above mentioned amino acids. Threonine and glutamic acid were present as traces before hydrolysis, while after hydrolysis traces of methionine and valine were also detected. The filtrate from the glucose salts medium also contained other amino acids. Beside the above amino acids, proline, valine, leucine, isoleucine, arginine, lysine, phenylalanine, tryosine and proteins appeared in the medium. The concentration of most amino acids increased after hydrolysis.

Table 2. Amino acids formed in culture media by *Penicillium decumbens* in uM/cm^2.

Amino Acid Detected	In Glucose Salt Medium		In Czapek Dox Medium	
	Before Hydrolysis	After Hydrolysis	Before Hydrolysis	After Hydrolysis
Alanine	0.0298	0.0370	0.0030	0.0060
Proline	0.0068	0.0135	—	—
Threonine	0.0197	0.0198	Trace	0.0018
Valine	0.0075	0.0116	—	Trace
Leucine	0.0106	0.0135	—	—
Serine	0.0073	0.0112	0.0030	0.0064
Glycine	0.0067	0.0254	0.0064	0.0103
Arginine	0.0109	0.0017	—	—
Lysine	0.0120	0.0147	—	—
Phenylalanine	0.0038	0.0086	—	—
Tryosine	0.0024	0.0006	—	—
Aspartic acid	0.0039	0.0028	0.0016	0.0070
Histidine	0.0034	0.0054	—	—
Glutamic acid	0.0127	0.0342	Trace	0.0060

Paper chromatography revealed the presence of glucose in the fungal filtrate from the glucose salts medium, while in Czapek Dox medium sucrose, glucose and fructose were detected.

Attraction of B. subtilis to Different Concentrations of Amino Acids

Attraction of *B. subtilis* to different concentrations (10^{-4} to 10^{-1}M) of the amino acids, L-asparagine, L-aspartate, L-leucine, L-proline and L-serine was

investigated. Figure 3 shows that leucine, serine and alanine were the best attractants, followed by asparagine and proline. Attraction of bacteria to aspartate was very weak, only 9.0×10^2 cells entered the capillary at the most favourable concentrations of the attractant. Other amino acids resulted in up to $4.7 - 9.9 \times 10^4$ bacteria entering the capillary (Table 3). The peak concentration of all the amino acids tested, apart from aspartate, was 10^{-2} or 10^{-3}M. The standard deviation was about 10% between replicate determinations at the favourable concentrations and may reach 15% at 10^{-1} or 10^{-4}M concentration.

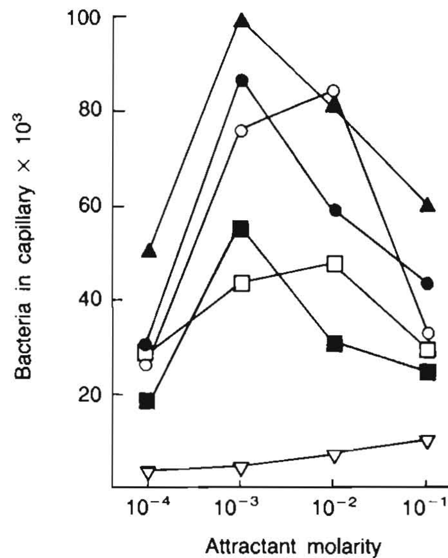

Fig. 3. Concentration-response curves for chemotaxis towards leucine ▲; alanine ○; asparagine ■; proline □; serine ● and aspartic acid △.

Table 3. Chemotaxis by *B. subtilis* towards some L. amino acids.

Attractant	Peak molarity (M)	Peak response (Bacteria in capillary)
Alanine	10^{-2}, 10^{-3}	82,000
Asparagine	10^{-3}	55,000
Aspartate	10^{-1}	9,000
Leucine	10^{-3}	99,000
Proline	10^{-2}, 10^{-3}	47,000
Serine	10^{-3}	87,000
Chemotaxis medium		1,600

Attraction of B. subtilis to Amino Acids at Different pH values

Table 4 shows that more bacteria were attracted to leucine (10^{-3}M) at low pH while serine (10^{-4}M) was not greatly affected by lowering the pH. The effectiveness of leucine at low pH is probably due partly to the fact that leucine at this concentration proved to be a very good attractant (Fig. 3) and partly to the use of citric acid which is responsible for the high values in the control experiment (Table 4).

Table 4. Attraction of *B. subtilis* to l- leucine, 10^{-3}M and l-serine, 10^{-4}M at different pH values.

Amino acid	No. bacteria attracted in capillary		
	pH 7.0	pH 5.0	pH 3.0
Chemotaxis medium	1,700	7,000	11,000
Leucine 10^{-3}M	90,000	92,000	99,000
Serine 10^{-4}M	28,000	24,000	23,000

Discussion

The requirements for the motility of the strain of *B.subtilis* (2745) used in the present work seem to differ from those of *B. subtilis* Marburg strain (60015) used by van der Drift and de Jong (1974). The motility of strain 2745 was optimum in the presence of the exogenous energy source, glucose, a chelating agent EDTA and $MgSO_4$. It did not require Tween 80 needed for the motility of the Marburg strain (60015). The requirements of strain 2745 and strain of *B. subtilis* 018 used by Ordal and Gibson (1977) were generally the same. However, magnesium sulphate was found to be essential for the motility of the *B. subtilis* 2745 strain used in this study. Ordal (1976) suggested that it is the local concentration of Mg^{2+} at the switch, that controls rotational direction of the flagella in *B. subtilis.* Glycerol was used as an energy source for both *B. subtilis* 60015 and *B. subtilis* 018, but for strain 2745 glucose afforded excellent motility.

The experiments reported here on the effect of time on chemotaxis showed that the highest numbers of bacteria in the capillary were obtained after 20 minutes followed by a slight decrease (Fig. 2). The subsequent decline in bacterial numbers was also observed for *B. subtilis* Marburg strain 60015 (van der Drift and de Jong, 1974). They considered this to be due to the aerobic nature of *B. subtilis.* Due to the relatively high oxygen concentration outside the tube, some of the bacteria tend to swim out, leading to the decrease in their numbers. The high motility of strain 2745 explains the short time needed to reach maximum numbers in the capillary.

P. decumbens used in the present work can form a large number of free amino acids and peptides in culture media within the 7-day incubation period. More amino acids were detected in the glucose salts medium than in the Czapek Dox medium. This is most probably due to the better growth in the former medium.

Some amino acids, *e.g.* alanine, threonine, leucine, arginine, lysine and glutamic acid, were present in comparatively high concentrations. No carbohydrate other than glucose was detected in the culture filtrate from the glucose salts medium. Verona *et al.* (1973), using the amino acid analyser were able to detect up to 20 free amino acids in fungal mycelia and culture media.

The above results showed that the fungal filtrate was a better source of attractant for *B. subtilis* than the original culture medium. The fungal filtrate and some amino acids were still good attractants at low pH (Tables 1 and 4) although low pH is known to be a repellent to a number of bacteria (Tso and Adler 1974, Seymour and Doetsch 1973). Strain 2745 being chemotactic towards low pH indicates that it either lacks taxis away from low pH or that the citric acid used in the preparation of the citrate phosphate buffer is a very good attractant to the bacterium and consequently its effect cancelled the repelling effect of low pH.

It has been demonstrated also in the present study that *B. subtilis* can be attracted in large numbers to a range of amino acids most of which could be exuded by *P. decumbens*. Production of amino acids, peptides, and possibly organic acids, minerals and vitamins by fungi (Cochrane 1966) in the culture medium were probably involved in making the filtrate more attractive to the bacterium. The concentrations of the individual amino acids released in the culture medium were generally very low. The highest concentration detected was 0.03 M per cm^3 filtrate for alanine. *B. subtilis* has been attracted to concentrations of 100 M per cm^3 of pure amino acids in the present work and would be expected to respond even to very small concentrations. The threshold concentrations were not determined in these cases. Ordal and Gibson (1977) have shown that their strain of *B. subtilis* responded to all 20 amino acids with thresholds varying from 3 nM for alanine to 0.1 mM for glutamate. The early work of Pfeffer (1888) has showed that a mixture of two attractants works better than either alone, even if one attractant is present in a very small concentration.

It is therefore assumed that, if fungi can exude similar compounds in soil, even in small quantities, motile chemotactic bacteria like *B. subtilis* could be attracted to them provided that a continuous water film exists.

Active movement of *B. subtilis* in soil has been demonstrated by Wong and Griffin, (1976a, b), and the movement of bacteria through the water film is considered as one of the principal ways by which bacteria obtain access to their food (Russel 1968). Fungi are continuously growing in soil and thus it would be expected that *B. subtilis* could detect nutrients released from the living hyphae, or

by-products of lysis and move towards them. Chemical attraction, therefore, is thought to be a factor that may cause accumulation of bacteria near fungal hyphae.

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إنجذاب بكتيريا *Bacillus subtilis* نحو المادة المفرزة بواسطة الفطريات ولمفردات الأحماض الأمينية في تلك المادة

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و

قسم علوم الحياة ـ جامعة اسمسيكمس ـ كولشستر ـ المملكة المتحدة

إتضح أن فطر *Penicillium decumbens* له المقدرة على تكوين كثير من الأحماض الأمينية الحرة والببتيدات والبروتينات في الأوساط الغذائية. إن انجذاب بكتيريا *Bacillus subtilis* نحو راشح الفطر المذكور وبعض الأحماض الأمينية قد تمت دراستها في هذا البحث. وقد وجد أن راشح الفطر المحتوي على المواد المفرزة يجذب ثلاثة أضعاف البكتيريا الذي يجذبه وسط المرق المغذي الأصلي. إن عدد البكتيريا المنجذب نحو التركيز المناسب للأحماض الأمينية Leucine, alanine serine في المدى ٨٠٢ ـ ٩٠٩ × ١٠^{٤} بينما عدد البكتيريا المنجذب بواسطة الأنابيب المعدة للمقارنة ١٠٦ × ١٠^{٣}. وقد وجد أيضاً أن راشح الفطر والأحماض الأمينية فعالان في جذب البكتيريا حتى عندما يكون تركيز أيون الهيدروجين عالياً. فإذا كان في مقدور هذه الفطريات أن تفوز في التربة مواد مشابهة للتي تفرزها في وسط المرق المغذي فإن البكتيريا المتحركة مثل *Bacillus subtilis* يمكن أن تتحرك نحو هذه المواد شريطة أن يكون هناك وسط مائي مستمر تتحرك فيه.