Motility and chemotaxis of a *Xanthobacter* sp. isolated from roots of rice

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Chemotaxis and substrate-regulated motility of the rice isolate *Xanthobacter* sp. JW-KR2 was studied. Growth on alcohols produced motile cells possessing several peritrichous flagella while growth in the presence of organic acids, such as succinate, repressed flagellar synthesis, leading to nonmotile cells. Addition of 2.5 mM-adenosine 3',5'-cyclic monophosphate (cAMP) to the culture resulted in motile cells even when succinate was the sole carbon source. Chemotaxis assays using microcapillary pipettes revealed a positive response to 1-propanol, 2-propanol, 1-butanol, 2-butanol and 1-pentanol but not to methanol, ethanol, isoamyl alcohol, hexanol, sugars, Casamino acids, tricarboxylic acid (TCA) cycle intermediates, butyrate, propionate, acetone or rice root exudates. The presence of TCA cycle intermediates in the chemotaxis assay inhibited chemotaxis towards butanol. Although the direct role of mobility and chemotaxis in the *Xanthobacter*–rice interaction is uncertain, one possibility is that *Xanthobacter* uses alcohols as a signal to move towards microaerobic zones, such as the rice rhizosphere, where carbon and energy sources such as H₂, CO₂, organic acids, alcohols and other anaerobic metabolites are present.

Introduction

Recently, *Xanthobacter* strains were isolated from the roots of wetland rice in Japan (Oyaizu-Masuchi & Komagata, 1988) and Arkansas, USA (Reding *et al.*, 1991). *Xanthobacter* sp. JW-KR1 multiplies in the rhizosphere of gnotobiotically grown rice and colonizes the root surface, resulting in a small benefit in plant top dry weight and number of leaves per rice plant after 41 d (Reding *et al.*, 1991).

One important factor in the colonization of a plant rhizosphere may be bacterial motility and chemotaxis (Ashby *et al.*, 1988; Gaworzewska & Carlile, 1982; Mellor *et al.*, 1987; Reinhold *et al.*, 1985). Because rice plants exude sugars, organic acids and amino acids from their roots into the surrounding rhizosphere (Boureau, 1977), a chemical gradient is formed towards which organisms may migrate. This gradient may in turn facilitate colonization of the rhizoplane by micro-organisms.

Until recently, only strains of *Xanthobacter agilis* were considered motile (Jenni & Aragno, 1987). Reding *et al.* (1992) found that motility of *Xanthobacter flavus* strains 301 and H4-14 was carbon-source-dependent. Strains of

Xanthobacter autotrophicus are considered nonmotile (Wiegel, 1991; Wiegel & Schlegel, 1984) although motile cells of *X. autotrophicus* 7cSF (a slime-reduced mutant of *X. autotrophicus* 7c) have been observed during very early exponential phase when cultured with propanol as the sole carbon source (Reding *et al.*, 1992). In this paper, we describe the requirements for motility and flagellation of *Xanthobacter* sp. JW-KR2 isolated from the roots of rice. In addition, we report on the unusual chemotaxis of *Xanthobacter* towards C₃ to C₅ aliphatic alcohols and the inhibition of chemotaxis toward butanol by the presence of TCA cycle intermediates.

Methods

Cultivation and maintenance of strains. *Xanthobacter* sp. JW-KR2 isolated from the roots of rice was used as a representative strain for the motility and chemotaxis experiments. Cells were cultivated routinely at 30 °C in medium A, a defined mineral salts medium (Wiegel & Schlegel, 1976). Medium A contains no organic carbon unless specified. Stock cultures were grown either on 80% (v/v) H₂, 10% (v/v) CO₂ and 10% (v/v) O₂, or 0.1% 1-propanol with an air atmosphere. Cultures were either kept at 4 °C for working stocks or at –80 °C in 50% (v/v) glycerol for long-term storage.

Growth rates and motility. Cells were cultured in sealed Hungate tubes containing 5 ml medium A (pH 6.8) with an appropriate carbon source (0.1%, w/v) and 15 ml air in the headspace. Cultures were routinely shaken at 140 r.p.m. in a model G25 incubator/shaker (New Brunswick Scientific). The sealed tube prevented evaporation of volatile carbon sources but still provided sufficient oxygen for growth. Growth was monitored by measuring OD₆₀₀. Chemicals were reagent grade (Sigma). To determine the percentage of motile cells, wet mounts

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Abbreviation: TCA, tricarboxylic acid.

were viewed under phase-contrast using an Olympus Vanox light microscope. Swimming rates were determined using a Zeiss phase-contrast microscope. Images were recorded in field mode with a Panasonic Super VHS cassette recorder using a Dage-MTI CCD72x camera.

Electron microscopy. Bacterial samples were negatively stained with 4% uranyl acetate using the single-carbon-layer technique of Valentine *et al.* (1968). Samples were examined using a JEOL 100CXII electron microscope at 80 keV.

Chemotaxis assays. Assays were conducted using microcapillary pipettes (Currier & Strobel, 1976). To produce motile, radiolabelled cells, *Xanthobacter* sp. JW-KR2 was grown on Tryptic Soy Agar (Sigma) at 30 °C for 3 d before being transferred into medium A containing 0.2% succinate as the carbon source and 100 µCi (3.7 MBq) [*trans*-³⁵S]methionine (1117 Ci mmol⁻¹ (41.33 MBq mmol⁻¹); ICN Biomedicals). At mid-exponential phase (around 1×10^8 cells ml⁻¹), the cells were centrifuged at 4000 g for 6 min under sterile conditions, then the pellet was washed and resuspended in an equal volume of medium A lacking a carbon source. This routinely caused 50–80% of the cells to become motile within 3 h. A 25 µl aliquot of the motile bacterial suspension was dispensed into an Eppendorf vial (0.5 ml capacity). A 5 µl microcapillary pipette was filled with the test compound dissolved in medium A, then the top of the pipette was heat-sealed and allowed to cool at room temperature. A single pipette containing the test compound in medium A or medium A alone was inserted into the bacterial suspension. After 90 min at 30 °C, the microcapillary pipette was removed, the exterior was rinsed with distilled water, and the contents were emptied into a scintillation vial containing Scintiverse BD cocktail (Fisher Scientific). Chemotaxis was quantified by counting each sample for 2 min with a Beckman model LS 5801 scintillation counter. The relative chemotactic response is expressed as the number of bacteria entering the microcapillary pipette containing the test compound divided by the number of bacteria randomly entering the control microcapillary pipette which contained medium A lacking any carbon source. Responses for three to five replicate samples were determined statistically using Duncan's Multiple Range Test ($P = 0.05$).

To test the ability of various carbon sources to inhibit chemotaxis of *Xanthobacter* JW-KR2, the test carbon source from a concentrated stock solution was added to a 25 µl motile cell suspension. Then, a microcapillary pipette containing 150 mM-butanol plus the test compound was inserted. After 90 min, the microcapillary tube was removed and processed as described above.

To collect rice root exudates aseptically, ten plants were cultivated in gnotobiotic assembly as described previously (Reding *et al.*, 1991). After 10 d, the plant growth medium (50 ml) was removed, lyophilized, and resuspended in 1 ml sterile distilled water. Glucose, fructose and sucrose concentrations were determined using a Combination Test Kit (Boehringer Mannheim).

Results and Discussion

Effect of carbon source and adenosine-3',5'-cyclic monophosphate (cAMP) on motility

Xanthobacter sp. JW-KR2 utilizes a wide range of carbon sources, including organic acids, amino acids, alcohols and some sugars (Reding *et al.*, 1991). Growth in medium containing methanol, ethanol, 1-propanol, 2-propanol, or butanol as the sole carbon source resulted in motile cells possessing two to five peritrichous flagella with a type I fine structure similar to *X. agilis* (Fig. 1;

Aragno *et al.*, 1977). Growth in medium containing citrate, fumarate, glutamate, glutamine, 2-oxoglutarate or succinate resulted in nonmotile, nonflagellated cells (data not shown). Other *Xanthobacter* strains isolated from rice (JW-KR1 to JW-KR6) behaved similarly. *Xanthobacter* sp. JW-KR2 utilized succinate with a doubling time of 3.5 h during mid-exponential growth phase (Fig. 2). These cells were nonmotile and lacked flagella as determined by electron microscopy (data not

Fig. 1. (a) Electron micrograph of a negatively-stained sample of *Xanthobacter* sp. JW-KR2 having several peritrichous flagella. Bar, 1 µm. (b) Type I flagellar fine structure similar to that of *X. agilis* (Aragno *et al.*, 1977). Bar, 20 nm.

Fig. 2. Growth (filled symbols) and percentage of motile cells (open symbols) during growth on 0.1% succinate (▲), 0.1% 1-propanol (●, ○), and 0.1% propanol plus 0.1% succinate (■, □; succinate was added to the propanol culture at 25 h after inoculation). Values represent the mean of 3–6 replicates.

Fig. 3. Relative chemotactic response (response relative to control lacking attractant) to various concentrations of (a) 1-propanol, (b) 2-propanol, or (c) 1-butanol. Values are the mean of 4 replicates $\pm$ SD.

shown). Upon transfer of the culture to medium containing 1-propanol as the sole carbon source 50% of the cells became motile with a slower doubling time of 5.5 h at mid-exponential growth phase (Fig. 2). When succinate was added to the cultures growing on propanol at 25 h after inoculation, the growth rate increased and the percentage of motile cells decreased to less than 1% within 6 h. The reduction of motile cells with the addition of succinate is likely to be due to repression of flagella synthesis. Motility of *Xanthobacter* sp. JW-KR2 is apparently induced by lack of quickly utilizable carbon in the medium. The downshift from succinate to 1-propanol as the carbon source resulted in a slower growth rate and synthesis of flagella. Flagella were also synthesized when cells grown in medium containing succinate or fumarate were transferred to medium lacking carbon. These cells became motile within 2 to 3 h of removal of the carbon source and remained motile for up to 32 h (data not shown). *Xanthobacter* spp. produce large amounts of poly- β -hydroxybutyrate as carbon storage, which can account for as much as 70% of the cellular dry weight (Wiegel & Schlegel, 1984). Presumably, *Xanthobacter* spp. are able to utilize the poly- β -hydroxybutyrate to synthesize flagella and become motile under carbon starvation.

In cultures of *Xanthobacter* sp. JW-KR2 grown on 0.1% succinate, the addition of 2.5 mM-cAMP produced motile cells with a slower growth rate, comparable to cells grown on 1-propanol alone (for typical growth

curves of succinate or propanol cultures, see Fig. 2). Growth and motility of cells cultured on 0.1% 1-propanol alone were not affected by the addition of 2.5 mM-cAMP. In this respect, the effect of cAMP on *Xanthobacter* sp. JW-KR2 is comparable to the effects observed with *Escherichia coli*. Flagellation of *E. coli*, and thus motility, is also carbon-source-dependent. In this case, the presence of glucose represses synthesis of flagella (Adler & Templeton, 1967). Addition of 2.5 mM-cAMP to medium containing glucose produces motile cells (Dobrogosz & Hamilton, 1971) with a slower growth rate (Mikheeva, 1985). The presence of cAMP allows the flagellar operon to be transcribed even in medium containing glucose (Silverman & Simon, 1974; Yokota & Gots, 1970). Surprisingly, the addition of cAMP to *Xanthobacter* sp. JW-KR2 cultures containing both succinate and 1-propanol neither decreased the growth rate nor resulted in the cells becoming motile (data not shown). The reason for this unexpected occurrence is unclear. *X. flavus* 301 behaved similarly with respect to cAMP and motility (data not shown).

Chemotaxis

Xanthobacter sp. JW-KR2 demonstrated unequivocal chemotaxis toward 1-propanol, 2-propanol and butanol between 15 mM and 500 mM (nominal concentrations in the capillary tubes; see below) with no peak response observed (Fig. 3). The lack of a peak response is rather atypical for a dose-response curve; however, control experiments using nonmotile cells did not mimic a positive response when up to 500 mM-alcohol was present in the microcapillary tube. This rules out false positive results due to density-dependent hydrodynamic effects which can occur if high concentrations are present in the microcapillary tube (Shonnard *et al.*, 1992). No response was detected to the alcohols between 1.5 mM and 0.0015 mM. Unequivocally positive responses (relative responses of 3.4, 3.6 and 8.9, respectively) were observed for 150 mM-pentanol, 15 mM-pentanol and 150 mM-2-butanol, but no positive responses were detected below these concentrations (down to 0.0015 mM; data not shown). The likely oxidation products of 1-propanol, 2-propanol and butanol (propionate, acetone and butyrate, respectively) did not produce a positive chemotactic response, indicating that the chemotaxis is specific for the alcohols. *Xanthobacter* sp. JW-KR2 showed no significant chemotaxis towards glucose, fructose, sucrose, methanol, ethanol, isoamyl alcohol, hexanol, citrate, fumarate, oxalate, 2-oxoglutarate, malate or succinate at concentrations between 0.0015 and 150 mM. Furthermore, no response was detected toward concentrated rice root exudates (glucose, fructose and sucrose concentrations of 4.4, 1.1, and 0.23 mM, respectively). To our

Table 1. *Substrate inhibition of chemotaxis to 150 mM-butanol*

Values represent the percentage ($\pm$ SD) of cells entering the capillary tube containing 150 mM-butanol plus the test inhibitor relative to the control (150 mM-butanol only). The cell suspension also contained a similar amount of the test compound so as not to create a second gradient.

Inhibitor	Concn (mM)				
	0	1.5	7.5	15	75
Citrate	100 $\pm$ 16	183 $\pm$ 73	125 $\pm$ 41	96 $\pm$ 12	17 $\pm$ 6*
Fumarate	100 $\pm$ 16	74 $\pm$ 31*	68 $\pm$ 38*	40 $\pm$ 4*	24 $\pm$ 4*
Succinate	100 $\pm$ 16	14 $\pm$ 5*	28 $\pm$ 9*	43 $\pm$ 4*	16 $\pm$ 4*
Glutamate	100 $\pm$ 16	85 $\pm$ 15	10 $\pm$ 4*	13 $\pm$ 4*	20 $\pm$ 8*
Glucose	100 $\pm$ 16	88 $\pm$ 15	86 $\pm$ 32	93 $\pm$ 14	105 $\pm$ 20
Sucrose†	100 $\pm$ 16	172 $\pm$ 30	112 $\pm$ 59	179 $\pm$ 24	120 $\pm$ 5

* A statistically significant decrease in response compared to the control as determined by Duncan's Multiple Range Test.

† Strain JW-KR2 does not utilize sucrose.

knowledge, this is the first report of bacterial chemotaxis towards aliphatic alcohols, although chemotaxis to aromatic alcohols (Ashby *et al.*, 1988) and sugar alcohols (Haneline *et al.*, 1991) has been reported. Other studies have reported negative chemotaxis, i.e. bacteria move away from alcohols (Tso & Adler, 1974; Smith & Doetsch, 1969). The only report of chemotaxis towards aliphatic alcohols is for zoospores of the phytopathogenic fungus, *Phytophthora cinnamomi*, which are chemotactic towards methanol, ethanol, n-propanol and n-butanol (Allen & Newhook, 1973). Ethanol is produced by some water-stressed plants (Bolton & Erickson, 1970). Therefore, Allen & Newhook (1973) postulated that ethanol produced by plant roots may encourage the accumulation of zoospores at the site of exudation.

Glucose is the major carbon source found in root exudates of rice, accounting for 62% of the total carbon quantified (Boureau, 1977). Organic acids, such as oxalic acid, succinate, aconitate and citrate, are found only in minor amounts. It is not known whether aliphatic alcohols are exuded from the roots of rice under field conditions. Root exudates are routinely analysed after lyophilization, thus any alcohol present would be lost in this process.

The chemotactic response toward aliphatic alcohols may have no direct function in attracting *Xanthobacter* spp. to the roots, but alcohols may serve as a signal for *Xanthobacter* spp. to move towards microaerobic environments containing anaerobic metabolites such as H₂, CO₂, organic acids and alcohols. These anaerobic fermentation products are the main carbon and energy sources for *Xanthobacter* spp. Most *Xanthobacter* spp. are very limited with respect to sugar utilization: some strains use no sugars, whereas some can use fructose and/or sucrose and mannose. The utilization of glucose requires a long adaptation period for most strains,

including JW-KR2 (Wiegel, 1991). Adler (1975) and Reinhold *et al.* (1985) reported chemotaxis to compounds which are not utilized for growth, suggesting that this may serve to place the organism in a nutritious environment. For *Xanthobacter* spp. aerotaxis to low pO₂ might accomplish the same goal; however, no banding of *Xanthobacter* sp. JW-KR2 cells was observed in wet mounts under cover slips, which indicates an absence of a strong aerotactic behaviour.

The presence of TCA cycle intermediates or glutamate in the cell suspension and microcapillary pipette inhibited the positive chemotactic response to butanol, whereas the non-utilizable substrate sucrose enhanced the response (Table 1). Although growth in the presence of these compounds eventually leads to repression of flagellar synthesis, microscopic examination of the cell suspension at the end of the 90 min chemotaxis assay revealed no decrease in the percentage of motile cells or the rate of swimming, which was $19 \pm 5 \mu\text{m s}^{-1}$ (8–18 body lengths s⁻¹) and $17 \pm 4 \mu\text{m s}^{-1}$ in the absence and presence of 7.5 mM-succinate, respectively. Therefore, the inhibition must be due to a reduction of the chemotactic response towards butanol. The presence of glucose or sucrose, which are not readily utilized as carbon sources, did not inhibit chemotaxis to butanol.

A similar inhibitory effects on chemotaxis was observed with cultures of *Vibrio furnissii* (Bassler *et al.*, 1991), where the presence of TCA cycle intermediates or compounds that feed into the TCA cycle inhibited chemotaxis to sugars and glutamine. Moreover, the degree of inhibition correlated with the oxidation rate of each TCA cycle intermediate. Bassler *et al.* (1991) suggested a link between catabolism and taxis in *V. furnissii* which may involve an interaction between the two systems. Our results suggest that a similar system may exist for *Xanthobacter* sp. JW-KR2.

Because *Xanthobacter* sp. JW-KR2 did not show chemotaxis toward lyophilized rice root exudates or pure compounds reported to be produced by the roots, the role of motility and chemotaxis in the *Xanthobacter*-rice association is uncertain. Alcohols to which this *Xanthobacter* is attracted are likely to occur in anaerobic rice paddy soils; however, to our knowledge, alcohol concentrations in the rhizosphere compared to the nonrhizosphere soil have not been investigated. Chemotaxis to alcohol concentrations above 100 mM are unlikely to be of biological significance since they are not expected to occur at this concentration in soil and sediments. However, an unambiguous positive chemotactic response was obtained at concentrations as low as 15 mM. Hazelbauer & Adler (1971) found that the concentration of solute in a capillary tube to which *E. coli* can respond in a chemotaxis assay is 200-fold lower than the actual concentration of the solute in the capillary tube. This is due to the design of the assay, in which diffusion out of the capillary tube forms a concentration gradient. Therefore, alcohol concentrations in the soil as low as 0.075 mM (15 mM in the assay tube) might be able to elicit a chemotactic response by *Xanthobacter* sp. JW-KR2. Concentrations of 0.1 mM-aliphatic alcohols are of biological significance. Such (and higher) concentrations have been found in sediment samples from lakes in the vicinity of Athens, GA, USA (unpublished results). Thus, we believe that the observed chemotactic response, despite the relative high concentrations required for an unambiguous response in the test assay used, is of biological significance.

Overall, the data imply that under carbon-limiting conditions (i.e. absence of quickly utilizable carbon and energy sources) chemotaxis to aliphatic alcohols may direct *Xanthobacter* spp. to zones in the soil where products of anaerobic metabolism are likely to be present. Once reaching that point, utilization of organic acids (TCA cycle intermediates) would inhibit further chemotaxis to alcohols and eventually repress flagellar synthesis, leading to nonmotile cells.

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