Metabolism is required for chemotaxis to sugars in *Rhodobacter sphaeroides*

Yehudit Jeziore-Sassoon,[†] Paul A. Hamblin,
Carolyn A. Bootle-Wilbraham, Philip S. Poole[‡] and Judith P. Armitage

Author for correspondence: Judith P. Armitage. Tel: +44 1865 275299. Fax: +44 1865 275297.
e-mail: armitage@bioch.ox.ac.uk

Microbiology Unit,
Department of
Biochemistry, University of
Oxford, South Parks Road,
Oxford OX1 3QU, UK

Chemotaxis towards carbohydrates is mediated, in enteric bacteria, either by the transport-independent, methylation-dependent chemotaxis pathway or by transport and phosphorylation via the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). This study shows that *Rhodobacter sphaeroides* is chemotactic to a range of carbohydrates but the response involves neither the classical methyl-accepting chemotaxis protein (MCP) pathway nor the PTS transport pathway. The chemoattractant fructose was transported by a fructose-specific PTS system, but transport through this system did not appear to cause a chemotactic signal. Chemotaxis to sugars was inducible and occurred with the induction of carbohydrate transport systems and with substrate incorporation. A mutation of the glucose-6-phosphate dehydrogenase gene (*zwf*) inhibited chemotaxis towards substrates metabolized by this pathway although transport was unaffected. Chemotaxis to other, unrelated, chemoattractants (e.g. succinate) was unaffected. These data, in conjunction with the fact that mannitol and fructose (which utilize different transport pathways) compete in chemotaxis assays, suggest that in *R. sphaeroides* the chemotactic signal is likely to be generated by metabolic intermediates or the activities of the electron-transport chain and not by a cell-surface receptor or the rate or mode of substrate transport.

Keywords: *Rhodobacter sphaeroides*, chemotaxis, bacteria, carbohydrate transport, phosphotransferase system

INTRODUCTION

The chemotactic systems in enteric bacteria and in the purple photosynthetic bacterium *Rhodobacter sphaeroides* are fundamentally different (Armitage *et al.*, 1990a; Armitage, 1992; Armitage & Schmitt, 1997). In enteric bacteria, chemical gradients are usually detected via methyl-accepting chemotaxis proteins (MCPs) which span the cytoplasmic membrane (Hazelbauer *et al.*, 1990). In response to a decrease in chemoeffector concentration, the cytoplasmic domain of the MCPs activates the histidine protein kinase, CheA, resulting in the auto-phosphorylation of CheA on a conserved histidine

residue (H48) (Hess *et al.*, 1988). An accessory protein, CheW, is absolutely required as a link between CheA and the MCPs (Conley *et al.*, 1989). CheA-phosphate phosphorylates CheY (a response regulator), which in turn interacts with the switch proteins of the flagellar motor, causing the bacterium to tumble. The signal is terminated by CheZ, which enhances the intrinsic auto-phosphatase activity of CheY (Blat & Eisenbach, 1994). Adaptation to the continued presence of a chemoattractant is mediated by CheB (a methylesterase) and CheR (a methyltransferase), which together control the level of methylation of the MCPs at conserved glutamate residues (Stock *et al.*, 1985; Yonekawa *et al.*, 1983).

A chemotaxis operon has been identified in *R. sphaeroides* containing homologues of the enteric *cheA*, *cheW*, *cheR* and two *cheY* genes, but lacking *cheB* and *cheZ* homologues (Ward *et al.*, 1995a). In contrast to the enterics, mutation of these chemotaxis genes in *R. sphaeroides* has little effect on chemotaxis to most chemoattractants (Hamblin *et al.*, 1997). Although two

[†]Present address: Institut für Allgemeine Mikrobiologie, Universität Bern, Baltzerstrasse 4, 3012-Bern, Switzerland.

[‡]Present address: School of AMS, University of Reading, Whiteknights, Reading RG6 6AJ, UK.

Abbreviations: MCP, methyl-accepting chemotaxis protein; PEP, phosphoenolpyruvate; PTS, phosphotransferase system.

mcp homologues have been identified in this *R. sphaeroides* chemotaxis operon, unlike the situation in *Escherichia coli*, *McpA* of *R. sphaeroides* is not the receptor for a specific chemoattractant but instead is involved in responses to a range of chemoattractants under aerobic conditions (Ward *et al.*, 1995b). The principal chemoattractants for this organism include weak organic acids, many of which are strong repellents for enteric bacteria. It has been shown that all known attractants for *R. sphaeroides* are either metabolites or simple ions that affect metabolism and that transport of the attractant is necessary to elicit a tactic response (Jacobs *et al.*, 1995; Ingham & Armitage, 1987). In addition, at least limited metabolism is required for responses towards ammonia and L-alanine (Poole & Armitage, 1989; Poole *et al.*, 1993).

Some chemotactic responses in enteric bacteria are not mediated by MCPs (Niwno & Taylor, 1982). A major MCP-independent system is the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) (Lengeler & Vogler, 1989). The PTS is a group translocation system consisting of membrane-bound protein kinases (enzymes II, EII) specific for the transport and concomitant phosphorylation of a wide range of carbohydrates (Saier, 1989, 1993; Saier *et al.*, 1990). EIIs are phosphorylated as a result of cytoplasmic phosphoryl transfer reactions catalysed by the soluble protein kinases enzyme I (EI) and HPr (histidine-containing protein), with PEP acting as the phosphoryl donor. EI and HPr are general PTS components, necessary for the uptake of all PTS carbohydrates except D-fructose. Fructose regulates the expression of its own HPr-like protein called pseudo-HPr or FPr (Geerse *et al.*, 1989), which has significant primary sequence homology to HPr and can substitute for HPr if constitutively expressed (Holzapfel *et al.*, 1990). Mutants of enteric bacteria in which the HPr protein was replaced by a constitutively expressed FPr were normal for transport and phosphorylation but they showed no chemotaxis to PTS substrates. The data suggest that the rate of phosphorylation during FPr-dependent transport was sufficient to allow transport but not chemotactic signalling. Chemotaxis towards PTS-dependent carbohydrates requires CheA, CheY and CheW but not the MCPs (Rowell *et al.*, 1995). The chemotactic signal is dependent on the rate of phosphorylation through the HPr-dependent PTS transport pathway; unphosphorylated EI enzyme can interact and inhibit the autophosphorylation of CheA (Lux *et al.*, 1995; Gröbl *et al.*, 1990; Lengeler & Vogler, 1989).

Saier and co-workers have shown that fructose is the only carbohydrate transported through a PTS in *R. sphaeroides*. This system is related to the FPr system identified in enteric bacteria, but with the EIII, FPr and EI domains located on a single protein (Wu *et al.*, 1990, 1991). As chemotaxis to carbohydrates in enteric bacteria appears to use either MCP-based or HPr-dependent PTS-based chemotactic signalling we investigated the chemotactic responses of *R. sphaeroides* to carbohydrates. By studying both transport mechanisms and

several early steps in carbohydrate metabolism, and relating our results to investigations of chemotaxis, we have attempted to make general comparisons between the chemotactic systems in *R. sphaeroides* and the MCP-independent systems in enteric bacteria. The role of metabolism in *R. sphaeroides* chemotaxis was also investigated using a mutation in glucose-6-phosphate dehydrogenase, a key enzyme in the Entner–Doudoroff and pentose phosphate pathways.

METHODS

Strains and growth conditions. *R. sphaeroides* WS8 was a gift from W. R. Sistrom (University of Oregon). *R. sphaeroides* WS8N was a spontaneous nalidixic-acid-resistant derivative of WS8 (Sackett *et al.*, 1990). *Escherichia coli* DH5 α was obtained from Gibco-BRL. *E. coli* JM109 λ pir and S17- λ pir were provided by R. Penfold (University of Queensland, Australia). Strains and plasmids used in this study are shown in Table 1.

R. sphaeroides was grown in liquid cultures at 30 °C under either aerobic conditions in the dark or in an illuminated anaerobic cabinet. The medium was either a modified Hutners (succinate) medium supplemented with 0.1 % Casamino acids or a minimal M22 medium (Armitage *et al.*, 1985), supplemented with either succinate or a carbohydrate such as fructose, glucose or mannitol at 2 g l⁻¹. Cells were harvested in late-exponential phase at a density of approximately 10⁹ cells ml⁻¹ and suspended in 10 mM HEPES/NaOH (pH 7.2). For anaerobic cells, the HEPES/NaOH buffer was sparged with nitrogen for at least 20 min. *E. coli* strains were grown in Luria–Bertani (LB) medium at 37 °C with appropriate antibiotics. For *E. coli*, antibiotics were used at the following concentrations; ampicillin, 100 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; chloramphenicol, 50 µg ml⁻¹. For *R. sphaeroides*, antibiotics were used as above, and also nalidixic acid at 20 µg ml⁻¹.

Chemotaxis and motility assays. Qualitative measurements of chemotaxis were performed in soft agar plates with plugs of attractant as described by Poole *et al.* (1993). Motile bacteria were washed in sparged 10 mM HEPES/NaOH (pH 7.2), to remove all traces of growth medium, and resuspended at a density of approximately 4 × 10⁹ cells ml⁻¹ in 0.25 % w/v Difco Bacto-agar made up in 10 mM HEPES/NaOH and containing chloramphenicol (50 µg ml⁻¹) to inhibit protein synthesis and growth around the attractant plugs. At this stage backgrounds of competitor chemicals at 100 mM were added to the agar if required. Hard agar plugs (2.5 % agar) containing 20 mM attractant were cut with a 5 mm cork borer and inserted into the soft agar/cell suspension. The bacteria were incubated in a Petri dish under aerobic dark or anaerobic light conditions. After approximately 2–4 h incubation, a chemotactic response was observed as a ring of accumulation around the hard agar plugs.

R. sphaeroides swarm plates were prepared from M22 media containing 0.22 % agar and 100 µM carbon source. Stationary-phase culture (5 µl) was pipetted onto the surface of the agar and the plates incubated aerobically in the dark or anaerobically in the light for 24–48 h, after which time the distance travelled by the growing cells up a concentration gradient created by metabolism was measured. Plug plates and swarm plates were scored as a qualitative measure of responses

Table 1. Strains and plasmids used

Strain or plasmid	Characteristic	Source/reference
<i>E. coli</i>		
DH5 α	General cloning strain	Gibco-BRL
JM109 λ pir	Cloning strain for pJP5603, derivative of JM109 with λ pir on chromosome	Gift from R. Penfold (Miller & Mekalanos, 1988)
S17-1 λ pir	Conjugative strain for pJP5603, λ pir and mobilizing factors on chromosome	Gift from R. Penfold (Penfold & Pemberton, 1992)
<i>R. sphaeroides</i>		
WS8	Wild-type chemotaxis strain	Gift from W. Siström
WS8N	Spontaneous nalidixic-acid-resistant derivative of WS8	This laboratory
JPA117	Derivative of WS8N with deletion of chemotaxis operon, Nal ^r	This laboratory
JPA131	Tn5 mutant of JPA117, Km ^r Nal ^r	This study
JPA142	<i>zwf</i> -deficient mutant of WS8N, Km ^r Nal ^r	This study
JPA141	<i>zwf</i> -deficient mutant of JPA117, Km ^r Nal ^r	This study
Plasmids		
pUC18	General cloning vector; Ap ^r	Messing (1983), Yanisch-Perron <i>et al.</i> (1985)
pJP5603	Mobilizable suicide vector, Km ^r	Gift from R. Penfold (Penfold & Pemberton, 1992)
pAUL57	pUC18 containing 9.5 kbp kanamycin-resistant <i>EcoRI</i> fragment from JPA131, Ap ^r Km ^r	This study
pAUL65	pUC18 containing 0.7 kbp <i>HpaI/EcoRI</i> fragment of pAUL57, Ap ^r	This study
pBM1	pJP5603 containing 0.4 kbp <i>EcoRI/BglII</i> fragment of pAUL65, Km ^r	This study

according to both the diameter of the rings and the intensity of the response.

The behaviour of individual tethered cells was analysed using computerized motion analysis. Cells from late-exponential-phase culture (1 ml) were harvested and resuspended in 100 μ l of 10 mM HEPES/NaOH buffer (pH 7.2) containing chloramphenicol (50 μ g ml⁻¹). The cells were tethered in a humidity chamber by incubating 9 μ l cell suspension with 3 μ l anti-flagellar antibody. After 30 min incubation the coverslip was loaded onto a flow chamber (Berg & Block, 1984) and equilibrated for 30 min with HEPES/NaOH (pH 7.2). The cells were observed by phase-contrast microscopy at $\times 1000$ magnification. HEPES/NaOH buffers (pH 7.2) containing known chemoeffectors were passed through the chamber and the behaviour of the tethered cells was analysed using a computerized motion analysis system (Seescan Electronics) described previously (Poole *et al.*, 1988). The rotational behaviour of each cell was determined by observing the position of the cell body every 0.06 s for 900 s. The data for 10–14 cells were averaged, smoothed and plotted.

The behaviour of free-swimming cells was measured by drawing a culture into an 0.05 mm deep optically flat microslide (Camlabs) which was sealed with vaseline. Cells were viewed at $\times 1000$ magnification and about 2000 cells tracked over a 165 s period using a real-time Hobson Bactracker system (Hobson Tracking Systems). The mean velocity (μ m s⁻¹) and stopping frequency (SPF, stops s⁻¹) of the

cells was measured for three periods of 165 s and the data averaged.

Transport assays. Cells were harvested at 10000 *g* for 10 min, washed and resuspended in nitrogen-sparged HEPES at a concentration of 10⁹ cells ml⁻¹. Samples were incubated under aerobic dark or anaerobic light conditions to deplete the cells of endogenous energy stores. Uptake was initiated by the addition of 0.5 μ Ci (18.5 kBq) U-¹⁴C-labelled solute to a final concentration of 4 μ M. If potential competitors were used, 100 μ M competitor substrate was added to the starved bacterial suspension just before the addition of the radio-labelled test solute. Samples (100 μ l) were filtered with suction through Whatman GF/F glass fibre filters (0.7 μ m retention) every 15–30 s, using a Millipore sampling manifold. Samples were washed immediately with 5 ml HEPES and placed into 3 ml Optiphase HiSafe scintillation fluid and radioactivity measured using a Beckman LS 5000TD scintillation counter.

For measurement of trichloroacetic-acid-precipitable material, representing the incorporation of the test substrate into cell material, a final volume of 1 ml cell suspension in HEPES was used. Assays were initiated by the addition of 4 μ M (0.5 μ Ci) ¹⁴C-labelled test substrate. Samples (100 μ l) were removed at intervals over a 15 min period and injected into 3 ml ice-cold trichloroacetic acid (10%, w/v). After 30 min incubation on ice, the samples were filtered through Whatman GF/F filters and radioactivity was measured as described above.

Preparation of cell fractions. Crude extracts, cytoplasmic fractions and membrane fractions were prepared as described by Daniels *et al.* (1988) with a few modifications. Cells were harvested, washed and resuspended in 25 mM Tris/HCl (pH 8.0), containing 0.5 mM DTT, 10 mM EDTA, 100 µg lysozyme ml⁻¹, 29 µg DNase ml⁻¹, 10 µg RNase ml⁻¹ and 15 mM MgCl₂ and ruptured by two passages through a French pressure cell (SLM AMINCO) at 69000 KPa maintained at 4 °C. Cellular debris was removed by centrifugation at 12000 *g* for 20 min. The cell-free supernatant was then centrifuged at 120000 *g* for 2 h, to separate the cytoplasmic and membrane fractions. All procedures were performed at 4 °C.

Enzyme assays. The phosphorylation activity of the PEP-dependent PTS was measured using an adaptation of the method of Saier *et al.* (1971). The phosphorylation reaction was carried out at 30 °C in a final volume of 200 µl and contained 25 mM Tris/HCl (pH 7.8), 1 M potassium fluoride, 100 mM DTT, 0.5 M PEP, 1 M MgCl₂, 2 mg protein membrane extract ml⁻¹ and a fourfold excess by volume of cytoplasmic extract. The reaction was initiated by the addition of 100 mM U-¹⁴C-labelled D-carbohydrate as the phosphate acceptor, at an activity of 7 µCi ml⁻¹; 20 µl portions were taken at time intervals and filtered through a stack of three DEAE-cellulose filters (Whatman DE 81 2.3 cm discs). Discs were allowed to dry for 30 min at room temperature and were then washed with distilled water. The filter stacks, which retain sugar phosphates, were then placed into 3 ml Optiphase HiSafe for scintillation counting.

Mannitol dehydrogenase (EC 1.1.1.67) activity was measured in a reaction mixture containing 0.2 mM NAD⁺, 10 mM mannitol, 1 mM DTT and 500 µg cytoplasmic extract ml⁻¹ in 50 mM Tris/HCl buffer at pH 8.9 and 30 °C. The conversion of mannitol to fructose was observed spectrophotometrically as the reduction of NAD⁺ at 340 nm.

Fructokinase (EC 2.7.1.4) reaction mixture contained 0.2 mM ATP, 10 mM MgCl₂, 10 mM fructose, 0.2 mM NADP⁺, 3 U phosphoglucose isomerase (EC 5.3.1.9), 3 U glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 500 µg cytoplasmic fraction ml⁻¹ at pH 8.0 in 50 mM Tris/HCl buffer. The isomerase converted fructose 6-phosphate to glucose 6-phosphate, which was then acted upon by the dehydrogenase. The concomitant reduction of NADP⁺ was followed optically at 340 nm.

Fructose-6-phosphate kinase (EC 2.7.1.11) was measured using a method from Fraenkel (1968). The reaction mixture contained 1 mM fructose 6-phosphate, 1 mM ATP, 5 mM MgCl₂, 5 mM DTT, 20 mM potassium phosphate buffer at pH 6.5, 0.15 mM NADH, 2 U fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), 2 U glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 2 U triose-phosphate isomerase (EC 5.3.1.1) and 500 µg cytoplasmic fraction ml⁻¹. The decrease in absorbance accompanying the oxidation of NADH was observed spectrophotometrically at 340 nm.

Fructose-1-phosphate kinase (EC 2.7.1.56) measurement was again adapted from a method of Fraenkel (1968). The reaction mixture contained 10 mM MgCl₂, 0.1 mM NADH, 1 mM fructose 1-phosphate, 2 mM ATP, 2 U fructose-1,6-bisphosphate aldolase, 2 U glycerol-3-phosphate dehydrogenase, 2 U triose-phosphate isomerase. The oxidation of NADH was observed spectrophotometrically at 340 nm.

Glucose-6-phosphate dehydrogenase reaction mixture contained 0.2 mM ATP, 10 mM MgCl₂, 0.2 mM NADP, 10 mM glucose 6-phosphate, 500 µg soluble protein extract ml⁻¹ in 50 mM Tris/HCl buffer. The formation of NADPH was

concomitant with the formation of 6-phosphogluconate. The reaction was carried out at 30 °C and pH 8.9.

Genetic techniques. Chromosomal DNA was prepared and plasmid preparations were performed by the Birnboim & Doly (1979) mini-prep technique or the Magic Miniprep system (Promega). Southern blotting was carried out using capillary transfer onto Hybond-N membranes and hybridized with probes generated using the random hexanucleotide priming method and digoxigenin-11-dUTP (Boehringer Mannheim). Hybridized fragments were visualized according to the manufacturer's immunodetection protocol. Standard cloning procedures were used throughout for the construction of plasmids. Constructs were transformed into *E. coli* DH5α, JM109 λpir or S17-1λpir using the calcium chloride method of Cohen (Sambrook *et al.*, 1989). *E. coli* JM109 λpir or S17-1 λpir were used to propagate plasmids pJP5603, pSUP2021 and their derivatives.

Sequencing reactions were performed on dsDNA by the dideoxy chain-termination method of Sanger using the Sequenase kit (Amersham) (Tabor & Richardson, 1987) and [³⁵S]dATPα-S (410 Ci mmol⁻¹, 15.2 TBq mmol⁻¹; Amersham). Samples were run on 6% polyacrylamide gels prepared using National Diagnostics Sequagel reagents. Oligonucleotide primers were prepared using β-cyanoethylphosphoramidite chemistry on an Applied Biosystems model 381A automated DNA synthesizer, deprotected overnight and precipitated using sodium acetate/ethanol and diluted to a working concentration of 5 pmol µl⁻¹. Primers used in this study (5' → 3') are as follows; Tn5SEQ, TCCTGGAAACGG-GAAAGG; pUC18FOR, GTTTTCCCAGTCACGAC; pUC-18REV, CAGGAAACAGCTATGAC.

Transposon mutagenesis of *R. sphaeroides* JPA117. JPA117 is a derivative of WS8N. An operon encoding homologues of the enteric chemotaxis proteins was deleted but the chemotactic phenotype of this mutant remained similar to wild-type (unpublished data). This strain, therefore, was used as the parent strain in an attempt to identify additional genes involved in sugar chemotaxis. pSUP2021, harbouring the transposon Tn5::Km was mobilized into *R. sphaeroides* JPA117 from *E. coli* S17-1 λpir as described by Simon *et al.* (1983a, b). Transposon mutants were selected on agar plates containing M22 medium, 7 mM succinate, 0.1% Casamino acids, nalidixic acid (20 µg ml⁻¹) and kanamycin (25 µg ml⁻¹). A total of 1000 single colonies were picked and grown independently and the cultures were tested for loss of chemotaxis using the swarm plate assay described above. The swarm plates were made from M22 medium containing 0.22% bactoagar, 100 µM mannitol and nalidixic acid (20 µg ml⁻¹) inoculated with 5 µl of stationary-phase culture and incubated aerobically. Transposon mutants were also plated directly into swarm agar, such that a large number of mutants could be rapidly screened. Reduced swimmers were selected and their chemotactic behaviour analysed. The Tn5 insertion site of a mutant which did not swarm to mannitol, JPA131, was cloned as a kanamycin-resistant 9.5 kbp *Eco*RI fragment into pUC18, generating plasmid pAUL57. A DNA section flanking the Tn5 was excised as *Hpa*I-*Eco*RI fragment from pAUL57, filled-in with Klenow and blunt-end cloned into the *Sma*I site of pUC18, generating pAUL65. pAUL65 was sequenced with primers Tn5SEQ and pUCFOR.

Insertional inactivation of the glucose-6-phosphate dehydrogenase gene (*zwf*). Plasmid pBM1 was used to insertionally inactivate the chromosomal *zwf* gene. It was constructed from a 0.4 kbp *Eco*RI-*Bgl*II fragment of pAUL65 ligated into the *Eco*RI/*Bam*HI site of the suicide vector pJP5603 (Penfold &

Pemberton, 1992). pBM1 was conjugated into *R. sphaeroides* WS8N and JPA117 from *E. coli* S17-1 λ pir and transconjugants were selected on kanamycin and nalidixic acid, generating strains JPA142 and JPA141 respectively. The interruption of the *zwf* gene was confirmed by Southern blotting using the 0.4 kbp *Eco*RI–*Bgl*II fragment as a probe.

RESULTS

Chemotaxis

R. sphaeroides (strain WS8) grew well, both heterotrophically and photoheterotrophically, on fructose, glucose and mannitol, and more slowly on mannose. A positive chemotactic response occurred to all these carbohydrates in plug plate assays (Table 2). Bacterial cells grown on succinate as the carbon source showed weak responses to some carbohydrates. If the cells were grown on the chemoattractant under test then the chemotactic response to that chemoattractant was strong. Thus the taxis to 20 mM glucose in a 2.5% (w/v) agar plug was stronger in cells grown on M22 medium with glucose as the sole carbon source. None of these carbohydrates were repellents, and indeed no repellents have been identified for *R. sphaeroides* (Armitage *et al.*, 1990b). Chemotaxis to organic acids, such as succinate, pyruvate, propionate or acetate, was equally strong in cells grown with either organic acids or carbohydrates as sole carbon sources (data not shown).

Transport

Rates of carbohydrate transport were higher if the bacteria were grown on the carbohydrate under test (Table 2). Thus the chemotactic response to carbohydrates in general correlated with the ability of cells to transport these substrates. Furthermore, sugar analogues, such as methyl α -D-glucoside or 2-deoxyglucose

were not transported and did not produce a tactic response (Table 2). This confirmed the results of Ingham & Armitage (1987) and subsequent work (Poole & Armitage, 1989; Poole *et al.*, 1993) which showed that the minimum requirement for chemotaxis to occur in *R. sphaeroides* was transport.

Incorporation

The rate at which the substrates were incorporated into intracellular trichloroacetic-acid-insoluble material following transport gave an indication of the rate at which the substrate was metabolized. Fructose was incorporated at a mean rate of 10 nmol (mg cell protein)⁻¹ min⁻¹ by fructose-grown cells; glucose was incorporated at 24 nmol (mg cell protein)⁻¹ min⁻¹ by glucose-grown cells; mannitol at 20 nmol (mg cell protein)⁻¹ min⁻¹ by mannitol-grown cells and mannose at 3 nmol (mg cell protein)⁻¹ min⁻¹ by mannose-grown cells. Succinate-grown cells showed rates of incorporation of approximately 1 nmol (mg cell protein)⁻¹ min⁻¹ for glucose, mannose and mannitol and a slightly higher rate of 4 nmol (mg cell protein)⁻¹ min⁻¹ for fructose. The values, representing the means of three experiments, correlate well with the mean transport rates shown in Table 2. The strength of the chemotactic responses obviously does not correlate directly with the absolute rate of transport and incorporation of individual sugars and this probably reflects the different metabolic pathways into which individual sugars go. This is supported by the data from the *zwf* mutant (see below).

The PEP-dependent PTS

Saier *et al.* (1971) showed that a fructose-specific PTS exists in purple photosynthetic bacteria and a detailed investigation of the PTS in *R. sphaeroides* strain 2.4.1

Table 2. Chemotaxis to and transport of attractants by *R. sphaeroides* pre-grown on succinate or on the attractant under test

Chemotaxis was measured in soft agar plates with agar plugs containing 20 mM attractant. The chemotactic response was observed as a band of accumulation around the hard agar plugs and was graded as follows: ++, strong; +, weak; 0, absent. Transport of attractant was measured as described in Methods, using 4 μ M attractant.

Attractant	Chemotactic response in cells pre-grown on:		Rate of transport of attractant [nmol (mg cell protein) ⁻¹ min ⁻¹] by cells grown on:	
	Succinate	Attractant	Succinate	Attractant
Succinate	++	++	25	25
Fructose	+	++	5	15
Glucose	0	++	1	28
Mannose	0	++	2	5
Mannitol	+	++	1	23
Methyl α -D-glucose	0	0	0	0
2-Deoxyglucose	0	0	0	0

Table 3. Phosphorylation of substrates by soluble and cytoplasmic extracts with either PEP or ATP as the phosphoryl donor

+ or – indicates the presence or absence of a component in the assay mixture.

Substrate	Component present in assay mixture				Rate of phosphorylation [nmol (mg cell protein ⁻¹) min ⁻¹]
	PEP	ATP	Membrane fraction	Cytoplasmic fraction	
Fructose	+	–	+	+	15.6
	–	+	+	–	<1.0
	–	+	–	+	2.0
Mannitol	+	–	+	+	0.0
	–	+	+	–	<1.0
	–	+	–	+	1.0
Glucose	+	–	+	+	0.0
	–	+	+	–	<1.0
	–	+	–	+	3.0

Table 4. Enzyme activities in the extracts of *R. sphaeroides* cells grown on different substrates under aerobic dark conditions

Abbreviations: PTS, phosphotransferase system; MDH, mannitol dehydrogenase; FK, fructokinase; F6PK, fructose-6-phosphate kinase; F1PK, fructose-1-phosphate kinase.

Growth substrate	Enzyme activity [nmol (mg cell protein) ⁻¹ min ⁻¹]				
	PTS	MDH	FK	F6PK	F1PK
Succinate	3.6	5.9	11.9	6.5	10.7
Fructose	11.6	6.0	13.1	11.6	20.6
Mannitol	10.1	14.3	22.6	5.0	28.4
Glucose	6.3	3.1	9.3	3.6	23.8

was carried out by Robillard (Lolkema & Robillard, 1985; Lolkema *et al.*, 1985, 1986). The data for *R. sphaeroides* WS8 corresponded well with previous investigations and showed that fructose was the only one of the carbohydrates under investigation which was phosphorylated by PEP. The phosphorylation only occurred in the presence of bacterial membrane and cytoplasmic extracts containing, respectively, EII and EI, essential for PTS function (Table 3). When ATP replaced PEP, the extent of fructose phosphorylation by fructose-grown cells was significantly lower and was primarily in the cytoplasmic fraction (Table 3) containing the ATP-dependent enzymes of sugar catabolism (Lolkema *et al.*, 1985).

Fructose PTS activity was found to be high in both fructose-grown and mannitol-grown cells, but low in glucose-grown and succinate-grown cells (Table 4). The PTS induced by growth on mannitol appeared to be the same as the PTS induced in fructose-grown cells. Both phosphorylated fructose at comparable rates (Table 4) and were fructose-specific (Table 3). Mannitol is converted into fructose intracellularly by mannitol de-

hydrogenase and this probably explains the ability of mannitol to induce the fructose PTS as well as its own transport system.

The role of the PTS in chemotaxis

A chemotactic response occurred to all the carbohydrates under investigation (Table 2). Only fructose, however, was transported through a PTS, the other carbohydrates utilizing different transport mechanisms. This suggests that in *R. sphaeroides* the chemotactic response is independent of the type of transport system.

Substrate competition experiments were carried out to identify whether transport alone could cause chemotactic signalling. Mannitol-grown cells were used as they had a high fructose PTS activity (Table 4) and also transported mannitol at high rates through another, non-PTS, transport system. If a saturating background of 100 mM mannitol was present in the chemotaxis plate, then the chemotaxis to 20 mM fructose was weaker and the incorporation of fructose was lower. The rates are presented as the means $\pm$ SD. Fructose was

incorporated at a lower rate of 3.75 ± 0.07 nmol (mg cell protein) $^{-1}$ min $^{-1}$ in the presence of a mannitol background, compared to 9.58 ± 0.74 nmol (mg cell protein) $^{-1}$ min $^{-1}$ with no background ($P = 0.000007$). The mean transport rate of fructose was also reduced, but only slightly, from 11.01 ± 0.86 nmol (mg cell protein) $^{-1}$ min $^{-1}$ to 9.36 ± 0.54 ($P = 0.0017$) in the presence of a mannitol background. P values were calculated using the non-parametric Mann-Whitney test and are presented as two-tailed comparisons. The difference between the incorporation rates was more significant than between the transport rates, suggesting that the weaker response might be due to the incorporation step rather than the transport.

Similarly a background of fructose reduced the mean rate of mannitol transport from 23.08 ± 1.86 nmol (mg cell protein) $^{-1}$ min $^{-1}$ to 15.11 ± 0.9 nmol (mg cell protein) $^{-1}$ min $^{-1}$ and the mean rate of incorporation from 20.51 ± 1.27 nmol (mg cell protein) $^{-1}$ min $^{-1}$ to 12.3 ± 0.56 nmol (mg cell protein) $^{-1}$ min $^{-1}$ and resulted in a weaker chemotactic response towards mannitol. Saturating backgrounds of either mannitol or fructose did not completely inhibit either the transport or chemotaxis to fructose or mannitol respectively, suggesting that although the sugars share some metabolic pathways, they may also use alternative pathways.

Enzyme activities

A range of enzymes involved in sugar catabolism were derepressed in *R. sphaeroides* after growth on different carbohydrates (Table 4). Growth on fructose induced the fructose PTS, fructose-6-phosphate kinase and fructose-1-phosphate kinase. Mannitol induced mannitol dehydrogenase and fructokinase suggesting that, as mannitol was not phosphorylated by the induced fructose PTS, it was transported via a non-PTS pathway and converted into fructose intracellularly. The higher levels of enzyme activity measured in mannitol-grown cells may reflect the higher levels of intracellular fructose resulting from the faster rate of mannitol transport.

Identification of a mannitol chemotaxis minus mutant

Previous attempts in this laboratory to identify a chemotaxis mutant of wild-type *R. sphaeroides* WS8N by Tn5 transposon mutagenesis were unsuccessful. We now attribute this effect to multiple chemosensory pathways which may compensate for mutations. To overcome this problem, JPA117, which lacks one set of chemotaxis genes but still shows sugar chemotaxis, was mutagenized with Tn5::Km and grown to single colonies on agar plates containing 7 mM succinate, 0.1% Casamino acids, kanamycin (25 µg ml $^{-1}$) and nalidixic acid (20 µg ml $^{-1}$). More than 1000 single colonies were picked and grown independently to stationary phase. Each colony was tested independently for swarming using 0.22% agar containing 100 µM mannitol. Reduced swimmers were identified and the swarm plate response

repeated three or four times until a stable phenotype was obtained. Only one mutant was obtained with a stable phenotype and this strain was labelled JPA131.

The sequence of the mutated gene showed 99% identity at the DNA level with the *zwf* gene from *R. sphaeroides* 2.4.1 (T. Conway, personal communication), confirming that the interrupted gene in JPA131 encodes the enzyme glucose-6-phosphate dehydrogenase.

To confirm the phenotype of JPA131 and to further characterize a mutation in the *zwf* gene, interruption mutations of the *zwf* gene in WS8N and JPA117 were created using the suicide vector pBM1, which contained an internal fragment of *zwf*. The chromosomal copy of *zwf* gene was insertionally inactivated by a single homologous recombination event. A Southern blot, using the 0.4 kbp insert of pBM1 as a probe, confirmed that the chromosomal copy of the *zwf* in WS8N and JPA117 had been disrupted (data not shown). These strains were labelled JPA142 and JPA141 respectively.

Phenotypic analysis of the *zwf* interruption mutant

Preliminary analysis of all the *zwf* interruption mutants (JPA131, JPA141 and JPA142) showed their chemotactic behaviour and metabolism to be very similar to, but distinct from, that of wild-type *R. sphaeroides* WS8N. Since JPA141 is deleted for the characterized chemotaxis operon of *R. sphaeroides* whilst JPA142 retains the operon, this suggests that the operon does not have a major role in the normal chemotactic response. A more subtle role in sugar chemotaxis was suggested in a previous study (Hamblin *et al.*, 1997). A detailed investigation of the phenotype of JPA142 was made.

The rate of mannitol transport was found to be the same in JPA142 and WS8N, 10.2 ± 2.4 nmol (mg cell protein) $^{-1}$ min $^{-1}$ and 11.6 ± 0.4 nmol (mg cell protein) $^{-1}$ min $^{-1}$ respectively. To ensure that the mannitol uptake and metabolic enzymes were fully induced, WS8N and JPA142 were grown on an M22-based medium supplemented with succinate, mannitol and Casamino acids.

The activity of glucose-6-phosphate dehydrogenase activity in WS8N and JPA142 grown on an M22 medium supplemented with succinate, mannitol and Casamino acids was 52 ± 1.9 nmol (mg cell protein) $^{-1}$ min $^{-1}$ and <1 nmol (mg cell protein) $^{-1}$ min $^{-1}$ respectively, confirming that the *zwf* gene had been insertionally inactivated.

Under aerobic or anaerobic conditions, JPA142 showed a 50–90% reduction on swarm plates compared with WS8N when the carbon source was 100 µM fructose, mannitol or glucose, although the most significant reduction in swarming was seen with JPA142 on 100 µM mannitol under aerobic conditions (Table 5). Swarming to weak organic acids such as acetate and propionate was unaffected.

A comparison of the plug plate responses of JPA142 and wild-type cells grown on a combined succinate/

Table 5. Comparison of swarm plate responses of WS8N and JPA142

Cells were inoculated onto 0.22% agar plates made from M22 medium, nalidixic acid (20 µg ml⁻¹) and 100 µM chemoattractant. Plates were incubated either aerobically or anaerobically for 1–3 d. +, Weak response; + + + + +, strong response, measured as both diameter and strength of swarm.

Chemoattractant	Swarm plate response of:	
	WS8N	JPA142
Aerobic		
Propionate	+ + + + +	+ + + + +
Fructose	+ + + + +	++
Glucose	+ + + + +	+++
Mannitol	+ + + + +	+
Anaerobic		
Propionate	+ + + + +	+ + + + +
Fructose	+ + + + +	+++
Glucose	+ + + + +	+++
Mannitol	+ + + + +	+/-

carbohydrate medium was also made. Under anaerobic conditions, JPA142 showed a significantly reduced chemotactic response to sugars such as fructose, mannitol and glucose, although the responses to propionate and oxygen were the same as the wild-type.

The behaviour of free-swimming cells was analysed using the Hobson Bactracker. WS8N had a mean swimming speed of $16.0 \pm 0.7 \mu\text{m s}^{-1}$ and a stopping frequency of $1.7 \pm 0.1 \text{ stops s}^{-1}$ while JPA142 had a mean swimming speed of $16.4 \pm 0.2 \mu\text{m s}^{-1}$ and a stopping frequency of $1.7 \pm 0.1 \text{ stops s}^{-1}$, confirming that there was no difference in free-swimming behaviour in unstimulated conditions. In the tethered cell analysis, anaerobic WS8N cells responded to a stepped increase in the mannitol concentration (0 to 2 mM) with a small increase in the probability of stopping, which adapted back to pre-stimulus behaviour within 3 min. JPA142 showed a very reduced and more transient response. The response of JPA142 to the addition and removal of 1 mM acetate was identical to that of the wild-type.

The growth rate of JPA142 was compared with that of WS8N on a variety of carbon sources. When grown on succinate medium (M22 medium containing 7 mM succinate and 0.1% Casamino acids), there was no difference in the growth rate between JPA142 and WS8N. In contrast, the growth of JPA142 on M22 medium containing either 7 mM glucose, fructose and mannitol was significantly inhibited unless Casamino acids were added to the growth medium. In the presence of 0.1% Casamino acids, however, JPA142 was able to grow aerobically extremely slowly on fructose (a doubling time of almost 20 h) but not at all on mannitol or

glucose. Anaerobically JPA142 could grow, if Casamino acids were present, on fructose, slowly on glucose and very slowly on mannitol.

DISCUSSION

Previous work with *R. sphaeroides* has suggested that transport and partial metabolism are required to produce a chemotactic response. Although a chemotaxis operon and *mcp* genes have now been identified in *R. sphaeroides* and there is evidence for multiple pathways, little is known about their function in chemosensing in *R. sphaeroides* (Ward *et al.*, 1995a, b). In *E. coli*, sugars are sensed using the MCP-dependent or PTS system which integrate at the histidine protein kinase, CheA (Hazelbauer *et al.*, 1990; Lengeler & Vogler, 1989; Rowsell *et al.*, 1995; Lux *et al.*, 1995). Here we investigated the chemotactic responses of *R. sphaeroides* to various carbohydrates and the role of metabolism in this process.

R. sphaeroides strain WS8 grew well on the four carbohydrates tested, in contrast to strain 2.4.1, which grew poorly (Szymona & Doudoroff, 1960). Fructose, but not the other carbohydrates, was transported through a fructose-specific PTS. *R. sphaeroides* showed strong chemotaxis towards a range of carbohydrates, but only if grown on them, suggesting that there are not constitutive MCP-like sugar receptors. Mannitol did not compete with fructose for transport through the fructose PTS system but did compete in chemotaxis assays. As mannitol is converted to fructose intracellularly, this suggests that chemotactic competition takes place at the level of metabolism rather than transport. This was in contrast to enteric bacteria, where the PTS has a definite function in chemotaxis (Grübl *et al.*, 1990; Postma & Lengeler, 1985; Taylor & Lengeler, 1990). There is no evidence for other carbohydrate transport systems being involved in chemotaxis in enteric bacteria.

The induction of a particular carbohydrate chemotactic system corresponded to the induction of the transport system and the catabolic pathways for that substrate. This suggests that the metabolic pathways induced or derepressed by growth on carbohydrates have a crucial function in chemotactic signalling to those substrates. Little is known about the metabolic pathways of *R. sphaeroides* or their change in activity under aerobic and anaerobic conditions, but the Embden–Meyerhof, Entner–Doudoroff and pentose phosphate pathways all operate and it seems probable from the data from the *zwf* mutant that the sugars feed into different pathways to different extents under different growth conditions. A saturating background of mannitol reduced the rate of fructose incorporation and also the strength of the chemotactic response to fructose, but did not completely inhibit either. Fructose transported by the PTS was concomitantly phosphorylated to fructose 1-phosphate and is then probably converted to fructose 1,6-bisphosphate by fructose-1-phosphate kinase. Mannitol was probably transported by a non-PTS mannitol transport system and converted into fructose intra-

cellularly by mannitol dehydrogenase, catabolized by a fructokinase to fructose 6-phosphate and converted to fructose 1,6-bisphosphate (Lolkema *et al.*, 1985). All these enzymes were present and active in *R. sphaeroides* WS8 grown on fructose, glucose and mannitol. It is likely that the intracellular competition for this catabolic pathway, shown by the considerably lower rate of fructose incorporation in the presence of a mannitol background, gave rise to the weaker chemotactic response.

JPA142 was originally isolated as a mutant in chemotaxis to mannitol. Sequence analysis of the Tn5 insertion site showed that the interrupted gene did not encode a sensory protein such as an MCP, but instead was a metabolic enzyme, glucose-6-phosphate dehydrogenase, a key enzyme in the Entner–Doudoroff pathway. Detailed analysis of this mutant showed that, although it was defective in chemotaxis towards mannitol and partially defective in chemotaxis towards glucose and fructose, its mannitol uptake and taxis to organic acids such as succinate, propionate and acetate were identical to the wild-type. The observation that the mutant could still grow under some conditions on glucose and fructose, and showed chemotaxis to these sugars, emphasizes the complexity of *R. sphaeroides* metabolism. The weak response to glucose and fructose and the differences in responses under aerobic and anaerobic conditions probably reflect the use of the Embden–Meyerhof pathway under these conditions and the partial by-passing of the Entner–Doudoroff pathway block caused by the *zwf* mutation, whereas mannitol must be exclusively metabolized through the Entner–Doudoroff pathway. This ability to channel sugars through different metabolic pathways is probably reflected in the different strengths of chemotactic responses under different conditions and the competition seen between sugars. The ability of JPA142 to show a tactic response correlates very closely with its ability to metabolize a particular chemoattractant.

The data presented here show that the chemotaxis to the carbohydrates is MCP-independent in *R. sphaeroides*. All the carbohydrates were chemoattractants despite being transported by different transport systems. Since the reduced tactic responses of JPA142, which carries a mutation in the glucose-6-phosphate dehydrogenase gene (Δzwf), can be attributed to a block in the Entner–Doudoroff metabolic pathway, we suggest that chemotactic responses to sugars in *R. sphaeroides* are sensed through metabolism and are independent of the mode of transport. The mechanisms by which metabolic activity might control chemotaxis are unknown, but recent data on *E. coli* show that fumarate levels altered by metabolic activity can directly control flagellar activity and it has been suggested that sensing the metabolic state may be an early form of chemotaxis (Montrone *et al.*, 1996). It is also possible that the chemotactic signal results from changes in the redox state of a component of the electron-transport chain, with NADH providing the link between sugar metabolism and electron transport, particularly as mannitol

elicits such a strong response and requires a dehydrogenase as the first metabolic step. Redox sensors such as the PrrA/PrrB two-component system, which is involved in the oxygen control of photosynthesis gene expression, are well characterized in *R. sphaeroides* (Eraso & Kaplan, 1994, 1995) and recent work in this laboratory has shown that redox sensing is involved in tactic responses to light and oxygen (Grishanin *et al.*, 1997). Current work is focused on the identification of the 'sensor' which may link metabolism and electron transport.

ACKNOWLEDGEMENTS

We would like to thank Dr Bruce Maguire for technical assistance and the BBSRC for grants to Y. J. S. and P. A. H. We are extremely grateful to Dr T. Conway for sharing sequence data from *R. sphaeroides* 2.4.1 prior to publication.

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Received 19 June 1997; revised 9 September 1997; accepted 19 September 1997.