***Agrobacterium radiobacter* and related organisms take up fructose via a binding-protein-dependent active-transport system**

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Washed cells of *Agrobacterium radiobacter* prepared from a fructose-limited continuous culture (D 0.045 h⁻¹) transported D-[U-¹⁴C]fructose in a linear manner for up to 4 min at a rate several-fold higher than the rate of fructose utilization by the growing culture. D-[U-¹⁴C]Fructose transport exhibited a high affinity for fructose (K_T < 1 μ M) and was inhibited to varying extents by osmotic shock, by the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and by unlabelled sugars (D-fructose/D-mannose > D-ribose > D-sorbose > D-glucose/D-galactose/D-xylose; no inhibition by D-arabinose). Prolonged growth of *A. radiobacter* in fructose-limited continuous culture led to the selection of a novel strain (AR100) which overproduced a fructose-binding protein (FBP) and showed an increased rate of fructose transport. FBP was purified from osmotic-shock fluid using anion-exchange fast protein liquid chromatography (FPLC). The monomeric protein (M_r 34 200 by SDS-PAGE and 37 700 by gel-filtration FPLC) bound D-[U-¹⁴C]-fructose stoichiometrically (1.17 nmol nmol FBP⁻¹) and with high affinity (K_D 0.49 μ M) as shown by equilibrium dialysis. Binding of D-[U-¹⁴C]fructose by FBP was variably inhibited by unlabelled sugars (D-fructose/D-mannose > D-ribose > D-sorbose; no inhibition by D-glucose, D-galactose or D-arabinose). The N-terminal amino acid sequence of FBP (ADTSVCLI-) was similar to that of several sugar-binding proteins from other species of bacteria. Fructose transport and FBP were variably induced in batch cultures of *A. radiobacter* by growth on different carbon sources (D-fructose > D-ribose/D-mannose > D-glucose; no induction by succinate). An immunologically similar protein to FBP was produced by *Agrobacterium tumefaciens* and various species of *Rhizobium* following growth on fructose. It is concluded that fructose is transported into *A. radiobacter* and related organisms via a periplasmic fructose/mannose-binding-protein-dependent active-transport system, in contrast to the phosphotransferase system used by many other species of bacteria.

Keywords: *Agrobacterium radiobacter*, fructose transport and metabolism, fructose-binding protein (FBP), binding-protein-dependent fructose transport

INTRODUCTION

The transport of fructose into Gram-negative bacteria usually occurs by a group-translocation mechanism catalysed by a fructose-specific phosphoenolpyruvate-

dependent phosphotransferase system (fructose-PTS) with the production of fructose 1-phosphate (see Dills *et al.*, 1980; Kornberg, 1990; Postma *et al.*, 1993).

In contrast, the transport of fructose into *Rhizobium* spp. and *Pseudomonas cepacia*, and also into mutants of *Xanthomonas campestris* lacking the fructose-PTS, results in the accumulation of unaltered sugar, i.e. via active transport rather than group translocation (Gardioli *et al.*, 1980; Allenza *et al.*, 1982; Glenn *et al.*, 1984; de Crecy-Lagard *et al.*, 1991). Furthermore, fructokinase and phosphoglucose isomerase required for the subsequent conversion of the

Abbreviations: FBP, fructose-binding protein; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FPLC, fast protein liquid chromatography; fructose-PTS, fructose-specific phosphoenolpyruvate-dependent phosphotransferase system; GBP, glucose-binding protein; LBP, lactose-binding protein

transported fructose to fructose 6-phosphate and hence to glucose 6-phosphate have been detected in *Rhizobium meliloti* (Gardioli *et al.*, 1980; Guezzar *et al.*, 1988), *R. leguminosarum* (Glenn *et al.*, 1984; McLaughlin & Hughes, 1989) and *P. cepacia* (Allenza *et al.*, 1982), and the loss of one or other of these enzymes results in a failure to grow on fructose. These observations thus indicate that fructose is taken up via a high-affinity, active-transport system and is then converted to glucose 6-phosphate prior to metabolism via the Entner–Doudoroff pathway known to be present in these organisms (Gardioli *et al.*, 1980; Glenn *et al.*, 1984; McLaughlin & Hughes, 1989). The nature of the fructose transport system has not been investigated in detail.

High-affinity, active transport is characteristic of various binding-protein-dependent sugar-transport systems which use ATP as a source of energy. *Agrobacterium radiobacter* and *Agrobacterium tumefaciens*, both members of the *Rhizobiaceae* family, transport glucose and galactose using binding-protein-dependent systems of this type, and *A. radiobacter* also transports xylose and lactose in this manner (Cornish *et al.*, 1988a, 1989; Greenwood *et al.*, 1990). Periplasmic binding proteins for glucose/galactose (GBP1), glucose/xylose (GBP2) and lactose (LBP) have been purified and characterized from these organisms (Cornish *et al.*, 1988a, 1989; Greenwood *et al.*, 1990; Williams *et al.*, 1992).

This paper describes the properties of a binding-protein-dependent transport system for fructose in *A. radiobacter*, together with the purification and properties of the fructose-binding protein (FBP) and the isolation of a novel strain which overproduces the transport system in response to prolonged growth in continuous culture under fructose limitation.

METHODS

Organisms. *Agrobacterium radiobacter* NCIB 11883 and *Agrobacterium tumefaciens* C58 were obtained from Dr J. Linton, Shell Research, Sittingbourne, Kent, UK and Dr V. Kleckner, University of South Bohemia, Ceske Budejovice, Czech Republic. *Rhizobium meliloti* and *Rhizobium leguminosarum* were obtained from Dr A. Downie, John Innes Institute, Norwich, UK. All strains were stored in 20% (v/v) glycerol at -20°C .

Growth conditions. Batch culture was carried out at 30°C in 500 ml baffled flasks containing 150 ml mineral salts medium (Cornish *et al.*, 1989) with fructose, glucose, mannose, ribose or succinate (2 g l^{-1}) as the carbon source. Fructose-limited fed-batch culture at an initial specific growth rate (μ) of 0.1 h^{-1} was carried out at 30°C essentially as described by Cornish *et al.* (1989) except that *Pseudomonas aeruginosa*, *R. meliloti* and *R. leguminosarum* were grown at 0.05 h^{-1} , and *R. meliloti* and *R. leguminosarum* were grown on minimal medium B⁻ as described by Spaink *et al.* (1992). Continuous culture ($D\ 0.045\text{ h}^{-1}$) was carried out at 30°C , pH 7.0, in mineral salts medium (Cornish *et al.*, 1988a) with fructose (2 g l^{-1}) as the growth-limiting nutrient using an LH series 500 chemostat with a 0.9 l working volume; the steady-state biomass under these conditions was $1.22\text{ g dry wt l}^{-1}$.

Preparation of washed cell suspensions. Washed cell suspensions ($5\text{ mg dry wt ml}^{-1}$) were prepared as described by

Cornish *et al.* (1988a). Broken cells were prepared by sonication on ice of a washed cell suspension ($4 \times 30\text{ s}$ at an amplitude of $12\text{ }\mu$).

Measurement of fructose uptake rates. Rates of fructose uptake were determined by measuring the incorporation of $250\text{ }\mu\text{M}$ D-[U- ^{14}C]fructose into cells suspended in 20 mM HEPES/KOH buffer, pH 7.0 ($1\text{ mg dry wt cells ml}^{-1}$), as described by Cornish *et al.* (1988a) for the uptake of glucose. Samples ($50\text{ }\mu\text{l}$) were taken at 15 s intervals over a period of 1 min and the cells collected by rapid filtration (filter pore size $0.45\text{ }\mu\text{m}$) and washing in 20 mM HEPES/KOH buffer, pH 7.0; the washed filters were immediately immersed in 4 ml OptiPhase HiSafe scintillation fluid and the radiation counted. For measurement of fructose uptake over periods $> 1\text{ min}$, the initial concentration of D-[U- ^{14}C]fructose was increased to 2 mM . For measurement of uptake rates at low concentrations of fructose ($1\text{--}25\text{ }\mu\text{M}$), the specific activity of the D-[U- ^{14}C]fructose was increased 10-fold [from $0.499\text{ }\mu\text{Ci }\mu\text{mol}^{-1}$ ($18.46\text{ kBq }\mu\text{mol}^{-1}$) to $4.99\text{ }\mu\text{Ci }\mu\text{mol}^{-1}$ ($184.6\text{ kBq }\mu\text{mol}^{-1}$)] and the cell density was decreased as required (minimum density $0.1\text{ mg dry wt cells ml}^{-1}$). The substrate specificity of the fructose transport system was determined by measuring the extent to which unlabelled sugars (10 mM or $250\text{ }\mu\text{M}$) reduced the rate of uptake of D-[U- ^{14}C]fructose after 10 s preincubation in the reaction mix prior to the addition of the radiolabelled substrate. The effect of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) ($20\text{ }\mu\text{M}$) was measured in a similar manner. The uptake of D-[U- ^{14}C]glucose and D-[U- ^{14}C]galactose by washed cells was carried out as for D-[U- ^{14}C]fructose.

Measurement of fructose accumulation. The accumulation of fructose was measured in 20 mM HEPES/KOH buffer, pH 7.0 ($1.0\text{ mg dry wt cells ml}^{-1}$), essentially as described by Cornish *et al.* (1988a) for D-[U- ^{14}C]glucose, but using D-[U- ^{14}C]fructose ($50\text{ }\mu\text{M}$) of 10-fold higher specific activity. Samples (0.2 ml) were withdrawn after 10 s, the cells collected by filtration, washed in 20 mM HEPES/KOH buffer, pH 7.0, and immediately immersed in boiling water (5 ml). Aqueous extracts prepared from five filters were pooled, lyophilized and the residue dissolved in water ($120\text{ }\mu\text{l}$). The resultant solution was analysed by HPLC using an Aminex HPX 87H ion-exclusion column (Bio-Rad) at a temperature of 30°C linked to an Anachem/Gilson chromatograph operating at a flow rate of 0.2 ml min^{-1} with $2\text{ mM H}_2\text{SO}_4$ as the solvent. Samples ($50\text{ }\mu\text{l}$) were collected at 15 s intervals and dispensed into 4 ml OptiPhase HiSafe scintillation fluid prior to measurement of radioactivity (the intracellular product was identified by comparing the retention time of the radioactive peak with that of fructose, fructose 6-phosphate and glucose 6-phosphate standards detected using a Bio-Rad refractive index monitor). For calculation of the accumulation ratio the intracellular volume was taken to be in the range $1\text{--}3\text{ }\mu\text{l}$ ($\text{mg dry wt cells}^{-1}$).

Osmotic shock. Washed cells were subjected to osmotic shock as described by Cornish *et al.* (1988a).

Purification of FBP. Washed cells prepared from a fructose-limited continuous culture of *A. radiobacter* ($D\ 0.045\text{ h}^{-1}$) were subjected to osmotic shock and periplasmic FBP was purified from the shock fluid using anion-exchange fast protein liquid chromatography (FPLC). Shock fluid was filtered through an acrodisc ($0.45\text{ }\mu\text{m}$ pore size) to remove particulate material, and the filtrate loaded on to a Mono-Q 10/10 column (Pharmacia) equilibrated with 20 mM bis-Tris buffer, pH 6.8. Proteins were eluted using a linear gradient of KCl ($0\text{--}250\text{ mM}$ over 30 min at a flow rate of 4 ml min^{-1}). GBP1, GBP2 and BP3 (Cornish *et al.*, 1988a) were identified, together with an additional protein which eluted at 86 mM KCl and was later shown to be FBP. The latter was essentially pure after this single purification step.

as shown using SDS-PAGE, and was frozen at -20°C until required. The native M_r of the protein was determined using two Superose 12 columns (Pharmacia) in tandem, pre-equilibrated with 20 mM bis-Tris buffer, pH 6.8, and eluted in the same buffer at a flow rate of 0.5 ml min^{-1} . The columns were calibrated with bovine albumin, M_r 66000 (monomer); egg albumin, M_r 45000; carbonic anhydrase, M_r 29000; and horse heart cytochrome c , M_r 12400.

Equilibrium dialysis. The binding constant and binding stoichiometry of the purified FBP for fructose was determined by equilibrium dialysis essentially as described previously for the binding of glucose to GBP1 and GBP2 (Cornish *et al.*, 1988a). This was performed using an eight-cell rotating module (Hofer Scientific Instruments) where each cell was divided into two chambers of 0.5 ml volume by a dialysis membrane (M_r cut off 6000–8000). Pure FBP (0.32 nmol) in 0.3 ml bis-Tris buffer, pH 6.8, was added to each of the chambers on one side of the module, and 0.3 ml bis-Tris buffer, pH 6.8, containing D-[U- ^{14}C]fructose at various concentrations in the range 0.25–10 μM was added to the chambers on the other side. The module was rotated at 10 r.p.m. for 24 h at 4°C to attain equilibrium, and samples (50 μl) were then taken in triplicate from each chamber and dispensed into 4 ml OptiPhase HiSafe scintillation fluid prior to measurement of radioactivity. The data were analysed by the method of Scatchard (1949). The amount of fructose bound (nmol nmol FBP $^{-1}$) was divided by the concentration of free fructose (μM) and plotted against the amount of fructose bound. K_D was equal to the reciprocal of the slope, and the stoichiometry of binding was equal to the intercept on the abscissa. The effect of unlabelled sugars on binding of D-[U- ^{14}C]fructose to FBP was measured by the same procedure but using 0.5 nmol pure FBP, 5 μM D-[U- ^{14}C]fructose and 200 μM unlabelled sugar.

Enzyme assays. Fructokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase activities were measured at 30°C as described previously by Gardiol *et al.* (1980), Guezzer *et al.* (1988) and Greenwood *et al.* (1990).

Preparation of antisera. Antisera to purified FBP were prepared as described previously for LBP (Greenwood *et al.*, 1990).

Other procedures. N-terminal amino acid sequencing of FBP was carried out by blotting an SDS-polyacrylamide gel containing the purified protein on to nitrocellulose, then eluting the protein on to a polyvinylidene difluoride disc, and sequencing with a model 470 gas-phase sequencer (Applied Biosystems). SDS-PAGE, Western blotting and estimation of protein in SDS-polyacrylamide gels were performed as described by Greenwood *et al.* (1990).

Chemicals. D-[U- ^{14}C]Fructose (290 mCi mmol $^{-1}$; 10.73 GBq mmol $^{-1}$), D-[U- ^{14}C]glucose (298 mCi mmol $^{-1}$; 11.03 GBq mmol $^{-1}$) and D-[U- ^{14}C]galactose (339 mCi mmol $^{-1}$; 12.54 GBq mmol $^{-1}$) were obtained from Sigma. All other chemicals were obtained from Sigma or BDH and were of the highest grade available.

RESULTS

Fructose uptake by A. radiobacter grown in continuous culture

Uptake of D-[U- ^{14}C]fructose by washed cells of *A. radiobacter* prepared from a fructose-limited continuous culture (D 0.045 h^{-1}) was linear for up to 4 min, prior to becoming slower but remaining linear (Fig. 1). The initial rate of fructose uptake [$29.9\text{ nmol min}^{-1}$ (mg dry wt

Fig. 1. Uptake of D-[U- ^{14}C]fructose by washed cells of *A. radiobacter* prepared from a fructose-limited continuous culture (D 0.045 h^{-1}). Washed cells were prepared and assayed for uptake in 20 mM HEPES/KOH buffer, pH 7.0 (1 mg dry wt cells ml^{-1}), as described in Methods.

cells) $^{-1}$; Table 1] was several times higher than the rate of fructose utilization by the growing culture (q_{fructose} ; $0.073\text{ g fructose h}^{-1}$ (g cells) $^{-1}$ [$= 6.8\text{ nmol min}^{-1}$ (mg dry wt cells) $^{-1}$]) suggesting that there was strong derepression of the fructose uptake system, presumably to offset the very low residual concentration of fructose in the culture during growth at low dilution rate under fructose limitation. Even higher uptake rates were obtained for D-[U- ^{14}C]glucose and D-[U- ^{14}C]galactose, indicating that the GBP1-dependent transport system for these substrates was also highly derepressed, presumably in an attempt to scavenge these sugars during fructose-limited growth. As no radiolabelled, non-metabolizable analogues of fructose were available, all further work was carried out using D-[U- ^{14}C]fructose.

The rate of D-[U- ^{14}C]fructose transport was not significantly altered by preincubating the washed cells with glycerol (10 mM) as an ancillary energy source, but was inhibited by $> 95\%$ following preincubation with the uncoupling agent FCCP (20 μM). The rate of transport remained essentially constant at fructose concentrations in the range of 1 μM –1 mM, indicating that the K_m (K_T) for fructose uptake was $< 1\text{ }\mu\text{M}$ and hence that the rate obtained with 250 μM fructose [$29.9\text{ nmol min}^{-1}$ (mg dry wt cells) $^{-1}$] was V_{max} .

Analysis of cell extracts prepared from washed cells which had been exposed to D-[U- ^{14}C]fructose (50 μM) for 10 s showed that fructose accounted for approximately 65% of the recovered radioactivity, with the remainder in the form of fructose 6-phosphate or glucose 6-phosphate (it was not possible to distinguish between these two sugar phosphates using the analytical procedures available). The accumulation ratio of D-[U- ^{14}C]fructose at this point, assuming an intracellular volume of 1–3 μl (mg dry wt cells) $^{-1}$, was in the range 22–67. Overall, these results

Table 1. Enzyme activities of *A. radiobacter* wild-type and AR100 grown in fructose-limited continuous culture (D 0.045 h⁻¹)

Uptake of D-[U-¹⁴C]fructose and D-[U-¹⁴C]glucose by washed cells, and the activities of other enzymes in broken cells, was measured as described in Methods. The values quoted are the mean of up to three independent determinations; the variation between independent experiments was < 10 %.

Enzyme activities [nmol min ⁻¹ (mg dry wt cells) ⁻¹]	Wild-type	AR100
Fructose uptake	29.9	45.6
Glucose uptake	80.7	62.5
Fructokinase	24.0	22.8
Phosphoglucose isomerase	451	338
Glucose-6-phosphate dehydrogenase	33.3	35.4

strongly suggested that *A. radiobacter* takes up fructose via a high-affinity active-transport system rather than via a fructose-PTS (which would not allow accumulation of unchanged fructose).

The growth of *Azorhizobium caulinodans* in batch culture with succinate as the source of carbon is accompanied by substantial alkalization of the growth medium (M. Evans & C. W. Jones, unpublished), as predicted from the known ability of this organism to take up succinate via a dicarboxylate transport system in co-transport with H⁺ (Ronson *et al.*, 1984). The possibility that fructose was transported into *A. radiobacter* via an analogous proton-symport system was therefore tested by measuring the pH of the medium during batch growth with fructose as the carbon source. No increase in external pH was observed, thus indicating that fructose was not taken up in co-transport with H⁺.

Exposure of washed cells of *A. radiobacter* to osmotic shock inhibited the rate of D-[U-¹⁴C]fructose transport by up to 30 %. SDS-PAGE and Western blotting using antisera to GBP1 and GBP2 showed that osmotic shock also caused the substantial release of several previously characterized, periplasmic sugar-binding proteins including GBP1 (M_r 36 500), GBP2 (M_r 33 500) and BP3 (M_r 30 500) (Fig. 2), whilst releasing only 1 % of the fructokinase activity which was used as a cytoplasmic marker (see below). GBP1 was present at a significantly higher concentration than in cells grown at the same dilution rate under glucose limitation (Cornish *et al.*, 1988a), whereas the concentrations of GBP2 and BP3 were essentially unchanged. No evidence was seen in these experiments for the presence of a novel protein which may have been associated with fructose transport. However, osmotic-shock fluid readily bound a significant amount of D-[U-¹⁴C]fructose (0.34 μ M) as measured by equilibrium dialysis, indicating that such a protein was present (albeit at low concentration) and hence that fructose is probably transported via a periplasmic, binding-protein-dependent system.

The substrate specificity of the fructose transport system was investigated by measuring the effect of a 40-fold excess of unlabelled sugars (10 mM) on the transport of D-[U-¹⁴C]fructose (250 μ M) by washed cells of *A. radiobacter* (Table 2). D-Arabinose had no effect on fructose transport,

and D-glucose, D-galactose and D-xylose (the GBP1- and/or GBP2-dependent transport systems for which are derepressed during fructose-limited growth; see above) inhibited fructose transport by $\leq$ 39 % (indicating that GBP1 and GBP2 are at most only marginally involved in fructose transport). In contrast, a 40-fold excess of D-fructose, D-mannose, D-ribose and L-sorbose all inhibited fructose transport by $\geq$ 83 % (D-fructose > D-mannose > D-ribose > L-sorbose). Furthermore, an equimolar concentration of D-mannose (250 μ M) inhibited D-[U-¹⁴C]fructose transport almost to the 50 % level obtained with unlabelled D-fructose (250 μ M), whereas equimolar concentrations of D-ribose and L-sorbose (250 μ M) inhibited D-[U-¹⁴C]fructose transport by only 9 % and 13 %, respectively (data not shown). These results strongly suggested that the fructose transport system is not specific to D-fructose, but also catalyses the transport of D-mannose (and, less effectively, D-ribose and L-sorbose).

Enzymes of fructose metabolism in *A. radiobacter*

Enzymic analysis of broken cells of *A. radiobacter* prepared from fructose-limited continuous cultures (D 0.045 h⁻¹) showed the presence of several enzymes which were potentially involved in the metabolism of transported fructose, viz. fructokinase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase (Table 1). All of these enzymes exhibited activities which were again sufficient to support the rate of fructose utilization by the growing culture [i.e. $\geq$ 24.0 cf. 6.8 nmol min⁻¹ (mg dry wt cells)⁻¹].

Effect of prolonged growth of *A. radiobacter* in fructose-limited continuous culture

After *A. radiobacter* had been growing in fructose-limited continuous culture (D 0.045 h⁻¹) for approximately 31 generations, a periplasmic protein of M_r approximately 34 000 (as measured by SDS-PAGE) started to increase in concentration and became maximally overproduced by about 39 generations (Fig. 3). SDS-PAGE and/or Western blotting of cell extracts using antisera to GBP1 and GBP2 showed that the overproduced protein was not GBP1, GBP2 or BP3, since the concentrations of GBP1

Fig. 2. Identification of sugar-binding proteins in osmotic-shock fluid from *A. radiobacter* grown in fructose-limited continuous culture (D 0.045 h^{-1}). Cellular proteins were separated using SDS-PAGE and either stained with Kenacid blue (a) or transferred on to nitrocellulose and probed with antisera to GBP1 (b) or GBP2 (c). Tracks: 1, M_r standards; 2, whole cells; 3, shocked cells; 4, shock fluid.

and BP3 remained essentially unchanged during the course of the experiment and the concentration of GBP2 fell slightly. In contrast, Western blotting using antiserum prepared against the purified FBP from *A. radiobacter* cross-reacted strongly with the overproduced protein,

Table 2. Effect of unlabelled D-fructose and other sugars on the uptake of D-[U- ^{14}C]fructose by washed cells of *A. radiobacter* prepared from a fructose-limited continuous culture (D 0.045 h^{-1})

Unlabelled sugars were present in a 40-fold excess over D-[U- ^{14}C]fructose (i.e. 10 mM cf. 250 μM). The values quoted are the mean of up to three independent determinations; the variation between independent experiments was < 10 %.

Addition	D-[U- ^{14}C]Fructose uptake rate (%)
None	100
D-Arabinose	103
D-Xylose	75
D-Galactose	67
D-Glucose	61
L-Sorbose	17
D-Ribose	10
D-Mannose	5
D-Fructose	0

and showed that the latter had approximately trebled in concentration.

After 61 generations the culture (designated culture A) was streaked out on nutrient broth-agar and grown at 30 °C for 48 h. One of the resultant colonies (designated strain AR100) was picked off and regrown in continuous culture under fructose limitation (D 0.045 h^{-1}). Washed or broken cells of AR100 exhibited similar or slightly lower D-[U- ^{14}C]glucose uptake, fructokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase activities cf. the wild-type organism (Table 1). In contrast the rate of D-[U- ^{14}C]fructose uptake was approximately 50 % higher than that of the wild-type organism, but this was still much less than the approximately threefold increase in the concentration of the FBP as indicated by the Western blotting.

Purification and properties of the FBP

The FBP was purified from an osmotic-shock fluid prepared from a fructose-limited continuous culture of *A. radiobacter* strain AR100. Anion-exchange FPLC yielded a homogeneous protein with an M_r of 34 200 as determined by SDS-PAGE and 37 700 as determined by gel-filtration FPLC, thus indicating that FBP exists as a monomer.

The capacity of FBP to bind D-[U- ^{14}C]fructose was determined using equilibrium dialysis. The resultant Scatchard plot (data not shown) revealed that FBP bound fructose at a single site (1.17 nmol fructose nmol FBP $^{-1}$) and with high affinity (K_D 0.49 μM). Subsequent competitive-binding assays using a 40-fold excess of unlabelled sugars over D-[U- ^{14}C]fructose (200 μM cf. 5 μM) showed that unlabelled D-fructose, D-mannose and D-ribose inhibited the binding of D-[U- ^{14}C]fructose by $\geq$ 95 %, whereas L-sorbose inhibited binding by only 54 % and D-glucose, D-galactose and D-arabinose had no

Fig. 3. Effect of prolonged growth of *A. radiobacter* in fructose-limited continuous culture (D 0.045 h^{-1}) on the concentration of FBP and other sugar-binding proteins. Cell samples were harvested at various intervals, then cellular proteins were separated using SDS-PAGE and either stained with Kenacid blue (a) or transferred on to nitrocellulose and probed with antisera to GBP1 (b), GBP2 (c) or FBP (d). Tracks: 1, M_r standards; 2, 12 generations of growth after attaining the initial steady-state; 3, 28 generations; 4, 31 generations; 5, 32 generations; 6, 39 generations; 7, 61 generations (culture A); 8, strain AR100 grown into steady-state continuous culture under the same conditions.

Table 3. Effect of unlabelled sugars on the binding of D-[U- ^{14}C]fructose to purified FBP

Unlabelled sugars were present in 40-fold excess over D-[U- ^{14}C]fructose (i.e. $200 \mu\text{M}$ cf. $5 \mu\text{M}$). The values quoted are the mean of up to three independent determinations; the variation between independent experiments was $< 10\%$.

Addition	D-[U- ^{14}C]Fructose binding (%)
None	100
D-Arabinose	103
D-Galactose	104
D-Glucose	110
L-Sorbose	46
D-Ribose	5
D-Mannose	0
D-Fructose	0

inhibitory effect (Table 3). It was concluded that FBP is a D-fructose/D-mannose-binding protein which also has a limited capacity to bind D-ribose and L-sorbose.

The N-terminal amino acid sequence of purified FBP was ADTSVCLI-. This sequence showed no similarity with the N-terminal sequences of GBP1 or LBP (Greenwood *et al.*, 1990; Williams *et al.*, 1992), but showed at least 25% identity (and 50% similarity when conservative replacements were included) with the N-terminal sequences of GBP2 (ADLVVGVS-; Cornish *et al.*, 1989) and of the

galactose- and ribose-binding proteins from *Escherichia coli* (ADTRIGVT- and KDTIALVV-, respectively) (Argos *et al.*, 1981). Furthermore, the absence of an N-terminal methionine from FBP (and from the other binding proteins) was commensurate with the loss of a signal sequence characteristic of a periplasmic location for the mature protein.

SDS-PAGE and Western blotting showed that purified FBP did not cross-react with antisera to GBP1 or GBP2, and that GBP1, GBP2 and LBP did not cross-react with antiserum to FBP (Fig. 4). Parallel dot-blot experiments also showed that FBP did not cross-react with antiserum to LBP (not shown). It was concluded, therefore, that FBP was immunologically distinct from the other sugar-binding proteins present in *A. radiobacter*.

Fructose uptake by *A. radiobacter* grown in batch culture

Uptake of D-[U- ^{14}C]fructose by washed cells of *A. radiobacter* grown in batch culture was variably induced by different carbon sources (fructose $>$ mannose/ribose $>$ glucose; no induction following growth on succinate) (Fig. 5). SDS-PAGE and Western blotting using antiserum to FBP confirmed that FBP was also induced similarly (fructose $>$ mannose/ribose $>$ glucose; no induction following growth on succinate) (Fig. 5). In all cases, fructose uptake rates and FBP concentrations were lower than in cells grown in fructose-limited continuous culture. Induction of fructokinase activity followed a dif-

Fig. 4. Immunological specificity of FBP. Purified FBP, GBP1, GBP2 and LBP were subjected to SDS-PAGE and either stained with Kenacid blue (a) or transferred on to nitrocellulose and probed with antisera to GBP1 (b), GBP2 (c) or FBP (d). Tracks: 1, M_r standards; 2, GBP2; 3, GBP1; 4, FBP; 5, LBP.

ferent pattern (fructose/mannose > glucose > ribose > succinate) to fructose uptake and FBP, but except following growth on succinate was equal to or greater than that following growth in fructose-limited continuous culture.

Screening of *Agrobacterium* spp., *Rhizobium* spp. and other species of Gram-negative bacteria for the presence of FBP

The presence of FBP in *A. tumefaciens*, *Rhizobium* spp. and various other species of Gram-negative bacteria, following growth in fructose-limited fed-batch culture to

Fig. 5. Induction of fructose transport and FBP in *A. radiobacter* by growth in batch culture on various carbon sources. Cellular proteins were separated using SDS-PAGE and either stained with Kenacid blue (a) or transferred on to nitrocellulose and probed with antisera to FBP (b). Tracks: 1, M_r standards; 2, D-fructose; 3, D-glucose; 4, D-mannose; 5, D-ribose; 6, succinate.

maximize induction of fructose transport, was investigated by SDS-PAGE and Western blotting using anti-serum to FBP (Fig. 6). Cross-reaction was observed with putative FBPs in *A. tumefaciens* (M_r 34 000), *R. meliloti* (M_r 36 000) and *R. leguminosarum* (M_r 33 500), but not in *Klebsiella pneumoniae*, *E. coli* or *P. aeruginosa*. Similar results were also obtained with cells grown in batch culture with fructose as the carbon source (not shown). It is therefore likely that *A. tumefaciens*, *R. meliloti* and *R. leguminosarum* transport fructose, at least following growth under fructose limitation, using an FBP-dependent uptake system similar to that present in *A. radiobacter*.

DISCUSSION

The results presented in this paper show that *A. radiobacter* grown in fructose-limited continuous culture takes up fructose via a high-affinity, active-transport system involving a periplasmic FBP, and not via either a fructose-PTS (as has been found in other species of bacteria; see Dills *et al.*, 1980; Kornberg, 1990; Postma *et al.*, 1993) or an H^+ -fructose symport system. The transported fructose is then converted to fructose 6-phosphate and thence to

Fig. 6. Screening of various species of Gram-negative bacteria for a protein capable of cross-reacting with antiserum to FBP from *A. radiobacter*. Organisms were grown in fructose-limited fed-batch culture (μ 0.05 or 0.10 h^{-1}), then cellular proteins were separated using SDS-PAGE and either stained with Kenacid blue (a) or transferred on to nitrocellulose and probed with antisera to FBP (b). Tracks: 1, M, standards; 2, *A. radiobacter*; 3, *A. tumefaciens*; 4, *R. meliloti*; 5, *R. leguminosarum*; 6, *K. pneumoniae*; 7, *E. coli*; 8, *P. aeruginosa*.

glucose 6-phosphate and 6-phosphogluconate before being further metabolized via the Entner-Duodoroff pathway (*A. radiobacter* has been previously shown not to exhibit phosphofructokinase activity, and hence not to metabolize sugars via the glycolytic pathway; Cornish *et al.*, 1988b). To our knowledge this is the first identification and characterization of a binding-protein-dependent transport system for fructose, although previous workers have reported active transport of fructose into *R. meliloti* and *R. leguminosarum* (Gardiol *et al.*, 1980; Glenn *et al.*, 1984).

The accumulation ratio for D-[U- ^{14}C]fructose of 22–67 was substantially lower than the accumulation ratio previously exhibited by this organism for the uptake of D-[U- ^{14}C]glucose via the GBP1/GBP2 systems (Cornish *et al.*, 1988a), but higher than that of [^{14}C]lactose via the LBP system (Greenwood *et al.*, 1990). It is likely, however, that the varied ratios reflect the different conditions under which they were measured (substrate concentration, time period) and the relative activities of the subsequent metabolic enzymes, rather than inherent differences in the energetics of the transport systems.

The fructose transport system was also active with mannose (and, to a lesser extent, with ribose), as

determined by measuring the effect of a limited range of unlabelled sugars on the binding and transport of radiolabelled fructose by FBP and washed cells, respectively. Purified FBP exhibited a high affinity for D-hexoses containing either a keto group (D-fructose) or a secondary alcohol group of the HOCH configuration (D-mannose) at C-2, and a low affinity for hexoses from which these groups were absent (D-glucose and D-galactose); the opposite was apparently true for pentoses, since FBP exhibited a higher affinity for pentoses which contained an HCOH group (D-ribose) rather than an HOCH group (D-arabinose) at C-2. In qualitative terms, fructose transport by washed cells exhibited a similar substrate specificity to fructose binding by FBP. However, D-glucose and D-galactose were quantitatively more effective against fructose transport than binding, a discrepancy which could possibly be explained by their ability to inhibit a putative outer-membrane porin for fructose (see Wylie & Worobec, 1993) rather than the binding-protein-dependent fructose transport system in the inner membrane.

It has previously been shown in this laboratory that the prolonged growth of *Agrobacterium* spp. in continuous culture at low dilution rate with glucose, galactose or lactose as the growth-limiting nutrient led to the selection

of novel strains which overproduced the relevant binding-protein-dependent transport system (Cornish *et al.*, 1988a, b, 1989; Williams *et al.*, 1990). Such strains thus increased their capacity for sugar transport, and hence exhibited an 'increased biological fitness' for growth under these conditions (Dykhuizen *et al.*, 1987) by increasing the rate at which they were able to transport sub-saturating concentrations of these sugars. The overproduction of GBP1 by *A. radiobacter* AR18 and *A. tumefaciens* AT18a during growth under glucose limitation, and of LBP by *A. radiobacter* AR50 during growth under lactose limitation, was accompanied by parallel increases in the rates of glucose/galactose and lactose transport, respectively, by washed cells (Cornish *et al.*, 1988a, b, 1989; Williams *et al.*, 1990). In contrast, overproduction of GBP2 by *A. radiobacter* AR9 and *A. tumefaciens* AT9 during growth under xylose limitation (Cornish *et al.*, 1989), and now the overproduction of FBP by *A. radiobacter* AR100 during growth under fructose limitation, was accompanied by much lower increases in the rates of glucose/xylose and fructose transport, respectively. It is possible, therefore, that overproduction of FBP and GBP2 by these novel strains was not accompanied by similar overproduction of the other components of the transport system.

The mechanism via which these changes may be brought about is not known but, by analogy with the genetic organization of the binding-protein-dependent lactose transport system in this organism (Williams *et al.*, 1992), could result from an up-promotor mutation giving a much higher concentration of FBP, followed by step-down for the other proteins in the system to a concentration only slightly higher than in the wild-type organism. The large increase in the concentration of FBP would enhance the rate at which fructose diffuses through the viscous periplasm (Brass *et al.*, 1986) and, together with the other changes, would thus cause the observed increase in the rate of fructose transport and hence endow strain AR100 with a significant selective advantage over the original wild-type organism. In this context it should be noted that GBP1 is not selected against during the course of the experiment, suggesting that the GBP1-dependent uptake system either transports fructose at a low rate or is expressed in response to starvation. The latter possibility is supported by the previous observation that GBP1 also remains derepressed during prolonged growth under lactose limitation (Williams *et al.*, 1990).

The non-parallel changes in the activities and/or concentrations of the fructose transport system and FBP compared with fructokinase during the isolation of strain AR100 suggested that the genes encoding the fructose transport system and fructokinase are not co-ordinately induced, and hence are not present on the same operon.

Previous work has shown that *R. meliloti* contains a moderately high affinity (K_m 22 μ M) active-transport system for mannose, which is then phosphorylated to mannose 6-phosphate by a specific mannokinase (Arias *et al.*, 1982). Furthermore, as we have now shown that *R. meliloti*, *R. leguminosarum* and *A. tumefaciens* all contain an

FBP which is immunologically similar to the FBP present in *A. radiobacter*, it is likely that fructose and mannose also share a common binding-protein-dependent transport system in all of these taxonomically related organisms.

The presence of very high-affinity ($K_m < 1 \mu$ M), binding-protein-dependent transport systems for fructose/mannose and many other sugars in *A. radiobacter* and related organisms (this paper; see also Cornish *et al.*, 1988a, 1989; Greenwood *et al.*, 1990), is fully commensurate with the natural habitat of these organisms in nutrient-poor environments. However, as these organisms grown in batch culture with fructose as the carbon source synthesize FBP and show no evidence of having either a fructose-PTS or an H^+ -fructose symport system, it would appear that they transport fructose using the high-affinity binding-protein-dependent system even during growth in the presence of excess fructose. This is in striking contrast to *E. coli*, which has more than one transport system for most sugars, and expresses the various systems differentially depending on the growth conditions (see Lendenmann & Egli, 1995).

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