Role of *sdhA* and *pfkA* and catabolism of reduced carbon during colonization of cucumber roots by *Enterobacter cloacae*

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We have been using a mutational approach to determine how plant-beneficial bacteria such as *Enterobacter cloacae* 501R3 obtain carbon and energy for colonization of subterranean portions of cucumber and other plants. Reduced carbon detected in cucumber root exudate consisted of 73.3 % amino acids, 22.2 % organic acids and 4.4 % carbohydrate. *Ent. cloacae* M2, a mini-Tn5 Km transposon mutant of strain 501R3, was severely reduced in *in vitro* growth relative to strain 501R3 on the mixture of amino acids and organic acids detected in cucumber root exudate when these compounds were supplied as the sole source of carbon and energy, but was similar in growth on the mixture of carbohydrates detected in this exudate. Molecular and biochemical characterization of *Ent. cloacae* M2 indicated that the transposon was inserted in *sdhA*, which encodes a subunit of succinate dehydrogenase. *Ent. cloacae* A-11, a mutant of strain 501R3 with a mini-Tn5 Km insertion in *pfkA*, was severely reduced in *in vitro* growth relative to strain 501R3 on the mixture of carbohydrates detected in cucumber root exudate, but similar in growth on the mixture of amino acids and organic acids. When strains A-11 and M2 were coapplied with strain 501R3 to cucumber seeds above carrying capacity in competitive root colonization assays, populations of strains A-11 and M2 were roughly one order of magnitude lower than those of strain 501R3 in cucumber rhizosphere, while populations of strains A-11 and M2 were similar to one other when coapplied to cucumber seeds. When *Ent. cloacae* strains were coapplied to cucumber seeds below carrying capacity, populations of A-11 and M2 were roughly two to three orders of magnitude lower than those of 501R3 in cucumber rhizosphere, and populations of A-11 were significantly lower than those of M2 when these two strains were coapplied to cucumber seed. The experiments reported here indicate an important role for *pfkA* and *sdhA* and the catabolism of carbohydrates, and of amino acids and organic acids, respectively, in the colonization of cucumber roots by *Ent. cloacae*. The results reported here also indicate that catabolism of carbohydrates by this bacterium is more important than catabolism of amino acids and organic acids at lower population densities, despite the much higher relative quantities of amino acids and organic acids detected in cucumber root exudate.

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Abbreviations: Ap, ampicillin; Cam, chloramphenicol; Kan, kanamycin; Rif, rifampicin; SDH, succinate dehydrogenase; TCA cycle, tricarboxylic acid cycle.

The GenBank/EMBL/DDBJ accession number for the sequence of the translated proteins of *Ent. cloacae* is AY194228.

INTRODUCTION

Applications of plant-beneficial bacteria as seed treatments for the suppression of plant diseases have been used to control root-infecting fungi, foliar pathogens, and soil-borne plant-parasitic nematodes (Weller, 1988; Larkin *et al.*, 1998; Meyer & Roberts, 2002). Colonization of subterranean plant parts by these beneficial bacteria is

thought to be essential for successful disease suppression in many applications (Lugtenberg *et al.*, 2001). Chin-A-Woeng *et al.* (2000) have provided compelling evidence that indicates a role for root colonization by *Pseudomonas chlororaphis* PCL1391 in suppression of *Fusarium oxysporum* f.sp. *radicis-lycopersici* on tomato. Others have also correlated colonization of plant surfaces with suppression of certain plant diseases (Bull *et al.*, 1991). Traits important for colonization of plant surfaces by beneficial bacteria include chemotaxis, prototrophy, putrescine uptake and stress tolerance, and the production of antibiotics, NADH dehydrogenases, site-specific recombinase and certain bacterial surface molecules (Mazzola *et al.*, 1992; Rainey, 1999; Lugtenberg *et al.*, 2001; de Weert *et al.*, 2002; Lohrke *et al.*, 2002; Martínez-Granero *et al.*, 2005).

Roots support growth and colonization by introduced beneficial bacteria and indigenous microbes through rhizodeposition (Lynch & Whipps, 1990). Soluble carbon compounds within rhizodeposits, which can represent between 1 and 40 % of total plant photosynthate (Whipps, 1990; Meharg, 1994), are thought to have the greatest stimulatory impact on growth and activity of microbes in the rhizosphere (Krafczyk *et al.*, 1984; Lynch & Whipps, 1990), influencing regions of the soil that extend millimetre distances radially from the root (Helal & Sauerbeck, 1983, 1986; Toal *et al.*, 2000). Sugars, amino acids and organic acids are the dominant soluble reduced carbon compounds contained within the complex mixtures of nutrients released from roots (Lynch & Whipps, 1990; Farrar *et al.*, 2003). The availability of these compounds is thought to limit the growth of introduced beneficial bacteria. Most carbohydrates, amino acids and organic acids are extremely labile in the rhizosphere due to consumption by the indigenous microflora and resorption by roots (Jones & Darrah, 1994b; Jones, 1998; Owen & Jones, 2001). The release and availability of these compounds to microbes in the rhizosphere vary spatially along the root (McDougall & Rovira, 1970; Chang & Roberts, 1989; Jones & Darrah, 1995; Jaeger *et al.*, 1999; Bringham *et al.*, 2001), with plant age, and with environmental conditions (Curl & Truelove, 1986; Grayston *et al.*, 1997).

Understanding the means by which introduced beneficial bacteria acquire the reduced carbon necessary for growth and colonization from the complex mixture of compounds released into the spermosphere and rhizosphere remains a challenge. *In vitro* techniques including the use of chemostats are available for the analysis of growth on low concentrations of mixtures of carbon compounds (Lendenmann *et al.*, 1996; Kovarova-Kovar & Egli, 1998). Methods for quantification of available reduced carbon components in rhizodeposits, and the dynamics of their release, are inadequate (Grayston *et al.*, 1997; Jones, 1998; Jaeger *et al.*, 1999), making representative *in vitro* studies of these compounds difficult. Consequently, spermosphere and rhizosphere colonization studies have been conducted with isogenic mutants of beneficial bacteria in which particular catabolic pathways have been made non-functional, allowing

the importance of particular pathways and compounds in rhizodeposits to be evaluated (Roberts *et al.*, 1999, 2000; Lugtenberg *et al.*, 2001; Lohrke *et al.*, 2002). Despite these studies, the contributing roles played by the catabolic pathways of beneficial bacteria, and the relative importance of carbohydrates, amino acids and organic acids within rhizodeposits to growth and colonization by beneficial bacteria, remain unclear (Roberts *et al.*, 1999).

Enterobacter cloacae is an enteric, plant-beneficial bacterium that suppresses damping-off caused by *Pythium ultimum* on cucumber and other crops (Hadar *et al.*, 1983; Nelson, 1988). *Ent. cloacae* colonizes the spermosphere and rhizosphere of several plant species, including cucumber (Roberts *et al.*, 1999; Lohrke *et al.*, 2002). Using strain A-11, an isogenic mutant of *Ent. cloacae* 501R3, it has been shown that catabolism of carbohydrates by this bacterium is important for colonization of seeds that release limited quantities of reduced carbon. Strain A-11 contains a transposon insertion in *pfkA*, which encodes phosphofructose kinase A, a key enzyme in glycolysis (Roberts *et al.*, 1999, 2000). M2 is a second prototrophic, transposon mutant of *Ent. cloacae* 501R3 that displays reduced colonization of seeds of cucumber and other crops (Roberts *et al.*, 1996). We report here that strain M2 contains a mutation in *sdhA*, a gene encoding a subunit of the tricarboxylic acid (TCA) cycle enzyme succinate dehydrogenase (SDH). The major objective of this study was to determine the relative importance of *pfkA* and *sdhA*, and consequently the relative importance of catabolism of carbohydrate and of amino acids and organic acids, to the colonization of cucumber rhizosphere by *Ent. cloacae* after this bacterium had been introduced into the planting medium above and below carrying capacity.

Bacterial populations are thought to reach carrying capacity when resource availability reaches equilibrium with resource use by that population in a given environment (Wilson & Lindow, 1994). Bacterial populations added above carrying capacity decline until the resources of the environment meet the resource needs of the population. In contrast, bacterial populations added below carrying capacity increase until carrying capacity is achieved. This equilibration of populations following the introduction of high and low numbers of bacteria has been observed in the spermosphere, rhizosphere and soil, and on leaf surfaces (Bennet & Lynch, 1981; Crozat *et al.*, 1982; Postma *et al.*, 1990; Wilson & Lindow, 1994; Roberts *et al.*, 1997a). We performed experiments above and below carrying capacity, as the relative fitness of bacterial mutant strains has been shown to vary with the ability of the environment to meet the resource needs of the bacterial population (De Leij *et al.*, 1998; Roberts *et al.*, 1999).

METHODS

Bacterial strains and plasmids. A description of the bacterial strains and plasmids is given in Table 1. *Ent. cloacae* strains A-11 and M2 are prototrophic mutants of *Ent. cloacae* 501R3 containing single

Table 1. Bacterial strains and plasmids

Abbreviations: Ap^r, ampicillin resistant; Cam^r, chloramphenicol resistant; Kan^r, kanamycin resistant; Rif^r, rifampicin resistant.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>Ent. cloacae</i> 501R3	Spontaneous Rif ^r mutant of <i>Ent. cloacae</i> EcCT501	Roberts <i>et al.</i> (1992)
<i>Ent. cloacae</i> A-11	Mini-Tn5 Km mutant of 501R3; <i>pfkA::</i> mini-Tn5 Km; Rif ^r , Kan ^r	Roberts <i>et al.</i> (1999)
<i>Ent. cloacae</i> M2	Mini-Tn5 Km mutant of 501R3; <i>sdhA::</i> mini-Tn5 Km; Rif ^r , Kan ^r	Roberts <i>et al.</i> (1996); this study
<i>E. coli</i> DH5 α	(Φ 80d <i>lacZ</i> Δ M15) Δ (<i>lacZYA-argF</i>) U169 <i>glnV-44 deoR gyrA-96 recA-1 relA-91 endA-1 thi-1 hsdR-17</i>	Sambrook & Russell (2001)
Plasmids		
pBeloBAC11	Cam ^r ; cloning vector	New England BioLabs
pGEM-7Zf(+)	Ap ^r ; cloning vector	Promega
pMK2	Ap ^r , Kan ^r ; contains 9.4 kb <i>KpnI</i> fragment from strain M2 containing <i>sdhCD</i> mini-Tn5 Km:: <i>sdhA</i>	This study
pMB7-1	Ap ^r , Kan ^r ; contains 6.3 kb <i>BglII</i> fragment from strain M2 containing mini-Tn5 Km:: <i>sdhA sdhB sucA</i>	This study
pGS133	Kan ^r ; contains <i>E. coli sdhCDAB</i>	Wood <i>et al.</i> (1984)
pBS4.5	Cam ^r ; contains 4.5 kb <i>BamHI</i> fragment from pGS133 in pBeloBAC11. Contains <i>E. coli sdhCDAB</i>	This study

mini-Tn5 Km insertions (Roberts *et al.*, 1996, 1999). Unless stated otherwise, strains were grown at 35 °C on Luria–Bertani (LB) broth (Miller, 1972) or agar, or M56 salts broth (Carlton & Brown, 1981) or agar. Broth cultures were shaken at 250 r.p.m. Culture media were supplemented with 50 μ g ml⁻¹ ampicillin (Ap), 25 μ g ml⁻¹ chloramphenicol (Cam), 75 μ g ml⁻¹ kanamycin (Kan) or 100 μ g ml⁻¹ rifampicin (Rif), where appropriate.

Molecular techniques. DNA isolation, transformation, electroporation, restriction digests, electrophoresis, ligation and Southern blot hybridization were performed as described elsewhere (Sambrook & Russell, 2001). Plasmid pMK2 was constructed by digesting *Ent. cloacae* M2 genomic DNA with *KpnI* followed by ligation to *KpnI*-digested pGEM-7Zf(+). Plasmid pMB7-1 was constructed by digesting *Ent. cloacae* M2 genomic DNA with *BglII* followed by ligation to *BglII*-digested pGEM-7Zf(+). Plasmids pMK2 and pMB7-1 were isolated after electroporation of these ligation mixtures into *Escherichia coli* DH5 α and selection on LB agar containing Ap and Kan. Plasmid pBS4.5 was constructed by digesting pGS133 with *BamHI* and ligating the resulting 4.5 kb *BamHI* DNA fragment to pBeloBAC11 that had been digested with *BamHI*.

DNA sequencing was carried out by PCR-mediated Taq DyeDeoxy terminator cycle sequencing (Applied Biosystems). Sequences were analysed using the DNA analysis programs of DNASTAR (Lasergene). BLAST searches of databases (Altschul *et al.*, 1997) were conducted with translated sequences by using the BLASTP program (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/BLAST). Alignments between *Ent. cloacae* and *E. coli* DNA sequences were conducted with DNA sequence available at www.Ecocyc.org (Keseler *et al.*, 2005). Pairwise identity and similarity of DNA and amino acid sequences were calculated with the BioEdit program version 7.0.5.3 (Hall, 1999). The PAM250 similarity matrix was adopted to calculate pairwise protein similarities.

SDH assay. *Ent. cloacae* strains were grown overnight in 250 ml Erlenmeyer flasks containing 25 ml LB broth supplemented with appropriate antibiotics. Cells from overnight cultures were harvested by centrifugation, washed with ice-cold 30 mM potassium phosphate, pH 7.6, centrifuged again, and resuspended in ice-cold 30 mM potassium phosphate, pH 7.6 (0.25 g cells ml⁻¹). One millilitre cell

suspension was sonicated (Vibra Cell sonicator with microtip, Sonics and Materials) six times for 10 s at a setting of 3.0. Cell debris was removed by centrifugation in a microcentrifuge at 4 °C for 1 min. Supernatant was transferred to a clean microcentrifuge tube and centrifuged at 4 °C for 10 min. This clarified supernatant was used for determinations of SDH activity and protein concentration. Reactions were performed at 22 °C, and SDH activity was determined spectrophotometrically at 455 nm (Beckman DU 520 spectrophotometer, 1 cm path length, Beckman Coulter), as described by Veeger *et al.* (1969). In this determination, 1 mol succinate reduces 2 mol K₃Fe(CN)₆. Protein concentrations were determined by the method of Bradford (1976) with BSA as standard using procedures recommended by the manufacturer (Bio-Rad Laboratories). One unit of specific SDH activity was defined as a decrease of 0.01 A₄₅₅ unit per minute per milligram protein. Experiments were performed twice with three replicates per treatment. The mean and SD of specific SDH activity were calculated for individual experiments.

Genetic restoration of growth on reduced carbon sources. *Ent. cloacae* strains 501R3, M2, M2(pBeloBAC11) and M2(pBS4.5) were streaked onto M56 salts agar plates containing 50 mM acetate, citrate, succinate, glutamine, proline or serine, or no carbon source, and incubated at 35 °C. The number of days of incubation required for formation of discrete colonies of each strain on each reduced carbon source was recorded. The experiment was performed at least twice with each reduced carbon source. Experiments were analysed independently.

Collection and characterization of cucumber root exudate. Cucumber (*Cucumis sativum* cv. Marketmore 76) root exudate was collected at 37 and 53 days from individual plants grown in 25 ml sterile nutrient solution (Hoagland & Arnon, 1950) in 250 ml Erlenmeyer flasks at 22 °C in a growth chamber with a 12 h photoperiod. Seeds were surface-sterilized, as previously (Roberts *et al.*, 1999), prior to use. Root exudate samples were checked for microbial contamination by spotting 10 μ l samples onto nutrient agar prior to analysis. Contaminated samples were discarded. Root exudate samples were passed through a 0.2 μ m pore-size filter and frozen until analysed.

Individual carbohydrates were determined by GC, essentially as previously (Sullivan & Schewe, 1977; Roberts *et al.*, 1997b), using an

Table 2. Reduced carbon compounds detected in cucumber root exudate

Values are mean $\pm$ SD of four replicate samples taken at 37 days and five replicate samples taken at 53 days. Means for individual carbohydrates, amino acids and organic acids at 37 and 53 days were not significantly different, so values from samples taken at these time points were combined prior to analysis. Note that two unidentified peaks eluted with retention times slightly different from those of ribose and certain disaccharides in root exudate samples.

Compound	Concn (μ g per plant)
Carbohydrates	
Arabinose	1.0 $\pm$ 2.5
Cellibiose	1.9 $\pm$ 2.4
Deoxyribose	BDT†
Fructose	0.5 $\pm$ 1.2
Fucose	BDT
Galactinol	BDT
Galactose	2.0 $\pm$ 3.1
Gentiobiose	0.8 $\pm$ 2.1
Glucose	5.2 $\pm$ 2.7
Isomaltose	0.3 $\pm$ 0.6
Lactose	0.5 $\pm$ 1.1
Maltose	0.4 $\pm$ 1.1
Mannitol	0.7 $\pm$ 1.8
Mannose	1.1 $\pm$ 2.7
Raffinose	BDT
Rhamnose	2.4 $\pm$ 3.8
Ribose	BDT
Sorbitol	BDT
Sorbose	BDT
Stachyose	BDT
Sucrose	11.8 $\pm$ 11.4
Trehalose	0.8 $\pm$ 1.9
Xylose	1.3 $\pm$ 3.2
Organic acids	
Citrate	12.7 $\pm$ 1.1
Isocitrate	7.0 $\pm$ 5.2
α -Ketoglutarate	46.3 $\pm$ 8.6
Malate	4.4 $\pm$ 0.4
Succinate	83.4 $\pm$ 17.5
Amino acids*	
Alanine	BDT
Arginine	5.1 $\pm$ 1.7
Aspartate	65.8 $\pm$ 51.7
Cysteine	BDT
γ -Aminobutyrate	0.9 $\pm$ 2.5
Glutamine	13.0 $\pm$ 2.5
Glycine	3.1 $\pm$ 6.0
Histidine	15.1 $\pm$ 18.4
Homoserine	BDT
Isoleucine	25.3 $\pm$ 27.3
Leucine	120.0 $\pm$ 119.2
Lysine	2.8 $\pm$ 0.3
Methionine	4.7 $\pm$ 3.8
Ornithine	0.5 $\pm$ 1.0
Phenylalanine	13.8 $\pm$ 28.5
Proline	188.1 $\pm$ 103.8
Serine	40.9 $\pm$ 23.4

Compound	Concn (μ g per plant)
Threonine	0.5 $\pm$ 0.9
Tryptophan	4.9 $\pm$ 4.5
Tyrosine	1.3 $\pm$ 0.2
Valine	BDT

*All amino acids reported here are the L stereoisomer.

†BDT, Below detectable threshold.

Agilent 5890 gas chromatograph and 5970 mass selective detector fitted with an RTX-200 column [105 m long, 0.25 mm internal diameter (ID), 0.25 μ m film thickness; Restek]. Carbohydrates were detected by selective ion monitoring at m/z 69. Individual amino acids and organic acids were derivatized as described by MacKenzie (1987) and analysed by GC using an Agilent 6890 chromatograph. An Agilent Ultra 2 column (25 m long, 0.2 mm ID, 200 μ m film thickness) was run at 1.0 ml min⁻¹ constant flow with hydrogen. Samples were injected at a split ratio of 100:1 and detected by electron capture. Samples were also run on an RTX-200 column (105 m long, 0.25 mm ID, 0.25 μ m film thickness; Restek) at 1.3 ml min⁻¹ constant flow with hydrogen, which gave better resolution of certain organic acids. Means for individual compounds at 37 and 53 days were not significantly different, so values from samples taken at 37 and 53 days were combined prior to analysis. Mean and SD were calculated from four replicate samples taken at 37 days and five replicate samples taken at 53 days for each compound.

In vitro growth assays. To determine growth on reduced carbon sources detected in exudate when supplied individually, *Ent. cloacae* strains cultured overnight in M56 salts broth plus 0.5 % glycerol, v/v, were harvested by centrifugation, washed twice with M56 salts without a reduced carbon source, and resuspended to OD₅₄₀ 1.00 (Spectronic Instruments model Spectronic 20D⁺ spectrophotometer, 1.8 cm light path) in M56 salts without a reduced carbon source, and 200 μ l of this suspension was used to inoculate 5 ml M56 salts broth plus 50 mM carbon source in 15 cm $\times$ 1.8 cm diameter test tubes. Cultures were incubated at 35 °C and 100 r.p.m. If there was no growth after 48 h incubation, glucose was added to 0.5 % final concentration and the culture incubated for an additional 8 h and tested for growth to ensure that the compound tested was not inhibitory. Growth of strains A-11 and M2 was considered substantially slower than that of strain 501R3 on a particular reduced carbon source if the OD₅₄₀ was 60 % or less than that of strain 501R3 at one or more time points. Growth by strains A-11 and M2 was considered slightly reduced if the OD₅₄₀ was 61–95 % that of strain 501R3 at one or more time points. Growth by strains M2 and A-11 was considered wild-type if the OD₅₄₀ was at least 95 % that of strain 501R3 at all time points. Experiments were performed at least twice with three replicate test tubes per strain for each test compound. Experiments were analysed independently.

Relative growth of *Ent. cloacae* strains was determined on synthetic cucumber root exudate (SRE-complete), or on the mixture of carbohydrates (SRE-CHO), or on the mixture of amino acids and organic acids (SRE-AA/OA) detected in cucumber root exudate. Experiments were performed as above, except that strains were incubated at 35 °C and 250 r.p.m. Carbohydrates, amino acids and organic acids detected in cucumber root exudate are listed in Table 2. For 0.5 % SRE-complete medium, the following compounds were added to 1380 ml M56 salts broth: 0.01 g L-arabinose, 0.019 g cellibiose, 0.005 g fructose, 0.02 g galactose, 0.008 g gentiobiose, 0.052 g glucose, 0.003 g isomaltose, 0.005 g lactose, 0.004 g maltose,

0.007 g mannitol, 0.011 g mannose, 0.024 g rhamnose, 0.118 g sucrose, 0.008 g trehalose, 0.013 g xylose, 0.051 g arginine, 0.658 g aspartate, 0.009 g γ -aminobutyric acid, 0.13 g glutamine, 0.031 g glycine, 0.151 g histidine, 0.253 g isoleucine, 1.20 g leucine, 0.028 g lysine, 0.047 g methionine, 0.005 g ornithine, 0.138 g phenylalanine, 1.881 g proline, 0.409 g serine, 0.005 g threonine, 0.049 g tryptophan, 0.013 g tyrosine, 0.127 g citrate, 0.07 g isocitrate, 0.463 g α -ketoglutarate, 0.044 g malate and 0.834 g succinate. For 0.5% SRE-CHO medium, the carbohydrates, in the quantities listed above, were added to 61.4 ml M56 salts broth. For 0.5% SRE-AA/OA medium, the amino acids and organic acids, in the quantities listed above, were added to 1316 ml M56 salts broth. Other concentrations of these media were prepared by diluting the 0.5% media with M56 salts broth. All media were filter-sterilized prior to use. Growth on these media was determined by measuring OD₅₄₀ periodically. Experiments were performed at least twice with each medium with three replicates per treatment. Experiments were analysed independently.

Root colonization assays. *Ent. cloacae* strains were grown overnight in LB broth, washed in sterile distilled water (SDW), and diluted in SDW to obtain the desired population density. Suspensions (40 μ l) containing strains 501R3, A-11, M2, 501R3+A-11, 501R3+M2 or A-11+M2 were applied to cucumber seeds. Seeds were sown (one seed per pot) in potting mix (Redi-Earth Peat Lite Mix, Scott's Horticultural Products) in 6.5 cm diameter $\times$ 25 cm deep pots and incubated in a growth chamber at 22 °C with a 12 h photoperiod. Plants were watered from above as needed throughout the experiment. At sampling time, the entire root system and tightly adhering planting medium were placed in SDW, sonicated for 5 min (model 8210, Branson Ultrasonics) and c.f.u. for each strain per root system determined by dilution plating. c.f.u. for strain 501R3 was determined by plating on M56 salts agar plus 0.2% *N*-acetylglucosamine plus Rif, or 0.2% proline plus Rif, c.f.u. for strain A-11 was determined by plating on M56 salts agar plus 0.2% proline plus Rif and Kan, while c.f.u. for strain M2 was determined by plating on M56 salts agar plus 0.2% *N*-acetylglucosamine plus Rif and Kan. Strain 501R3 cannot grow in the presence of Kan, while strains A-11 and M2 cannot grow with *N*-acetylglucosamine and proline as the source of reduced carbon, respectively. Mean log₁₀(c.f.u.) per root system was determined and compared using Least-squares Means in proc GLM (SAS Institute). Experiments were performed twice at each initial

population level with six replicates per treatment and analysed independently ($n=6$).

RESULTS

Molecular and biochemical characterization of *Ent. cloacae* M2

Ent. cloacae M2 was selected for further study after previous experiments had demonstrated this strain to be reduced in cucumber seedling colonization and to be significantly impaired in growth on amino acids (Roberts *et al.*, 1996). Southern hybridization analysis had also demonstrated the presence of a single mini-Tn5 Km insertion in the chromosome of strain M2 (Roberts *et al.*, 1996). Sequencing outward from mini-Tn5 Km in plasmids pMK2 and pMB7-1 (Table 1, Fig. 1), using primers specific for the ends of mini-Tn5 Km, indicated that this transposon was inserted in a region of the strain M2 genome with a high degree of similarity to *sdhA*, which encodes the flavoprotein subunit of SDH. Analysis of the combined DNA sequences from the *Ent. cloacae* portions of pMK2 and pMB7-1 revealed five ORFs with high similarity to *sdhCDAB* and *sucA* (Fig. 1). Each of the four ORFs for *sdhCDAB* contained a predicted ATG start codon and TAA stop codon, and was sufficient to encode polypeptides of 159, 115, 572 and 238 amino acids, respectively. *sdhCDAB* collectively encode all subunits of SDH (Keseler *et al.*, 2005). A partial sequence of *sucA* was obtained that contained all but the terminal nine amino acid codons of this gene. *sdhCDAB* and *sucA* have an identical organization and direction of transcription in *Ent. cloacae* and *E. coli* K12 (Keseler *et al.*, 2005). Comparison of this 6771 bp sequence from *Ent. cloacae* with that from *E. coli* K12 indicated an overall 84.9% DNA sequence identity.

Fig. 1. Physical maps of the portion of the *Ent. cloacae* M2 genome containing mini-Tn5 Km, and of plasmids pMK2 and pMB7-1. The location of genes and direction of transcription are indicated by horizontal arrows. The box indicates the site of the mini-Tn5 Km insertion. Kan^r, location of gene encoding Kan resistance; *Bgl*II and *Kpn*I, cleavage sites for restriction endonucleases *Bgl*II and *Kpn*I, respectively. *sdhCDAB* encode SDH subunits CDAB, respectively. *sucA* encodes oxoglutarate dehydrogenase subunit A. Features are approximately to scale.

To confirm that the mini-Tn5 Km insertion in *sdhA* in strain M2 affected SDH activity, an enzymic assay was performed. Specific SDH activity in cell lysates from *Ent. cloacae* M2 was 0.24 ± 0.01 units, approximately 30 % of the SDH activity in lysates from the parental strain 501R3. Specific SDH activity in cell lysates from *Ent. cloacae* 501R3 was 0.77 ± 0.02 units. Introduction of pBS4.5, containing *sdhCDAB* from *E. coli*, into strain M2 resulted in a 6.1-fold increase in SDH activity relative to strain M2. Cell lysates from M2(pBS4.5) had 1.47 ± 0.02 units of specific SDH activity. Strain M2 containing the empty cloning vector pBeloBAC11 had 0.23 ± 0.02 units SDH activity, equivalent to that of strain M2 alone. Results from a second experiment were similar (data not shown). It is possible that the SDH background activity in the *sdhA* mutant M2 is due to fumarate reductase. Many enteric bacteria contain both SDH and fumarate reductase, and fumarate reductase can catalyse succinate oxidation as measured with the $K_3Fe(CN)_6$ method used in this study (Hirsch *et al.*, 1963; Cecchini *et al.*, 2002). Fumarate reductase is typically produced in enteric bacteria at low oxygen tensions (Jones & Gunsalus, 1985).

Strain M2 did not grow on agar plates containing M56 salts plus 50 mM acetate, succinate, glutamine, proline or serine. All of these reduced carbon compounds supported growth by strain 501R3. Strain M2 was reduced in growth relative to 501R3 on M56 salts agar plus 50 mM citrate. As with strain M2, *sdh* mutants of *E. coli* were deficient in growth on acetate and succinate (Herbert & Guest, 1971). The growth capabilities of *E. coli sdh* mutants on citrate and the amino acids glutamine, proline and serine have not been reported (Herbert & Guest, 1971). The introduction of *E. coli sdhCDAB* on pBS4.5 into M2 completely restored growth relative to strain 501R3 on citrate, succinate and proline, and increased growth on acetate, glutamine and serine relative to strain M2. Plasmid pBeloBAC11, the cloning vector, did not result in increased growth by strain M2 on any compound tested. None of the strains grew on M56 salts agar without a source of reduced carbon. Results from a second experiment were similar (data not shown).

Analysis of cucumber root exudate

The complex mixture of reduced carbon compounds detected in cucumber root exudate contained substantially more amino acids and organic acids than carbohydrate (Table 2). Amino acids, organic acids and carbohydrates formed 73.3, 22.2 and 4.4 %, respectively, of the reduced carbon. Although variable between replicates, substantial amounts of the amino acids aspartate, leucine, proline and serine were detected, forming 9.5, 17.4, 27.2 and 5.9 % of the total reduced carbon, respectively. Substantial amounts of the organic acids succinate and α -ketoglutarate were also present, forming 12.1 and 6.7 % of the total reduced carbon detected, respectively. Glucose and sucrose were the most prominent carbohydrates released from cucumber roots. It is possible that the quantities of organic acids

relative to those of sugars and amino acids in root exudates were overestimated for the following reasons. (1) Plant roots in nutrient solution have been shown to resorb over 90 % of the sugar-carbon previously released into the nutrient solution (Jones & Darrah, 1993), and proteinaceous amino acids have been shown to be resorbed by roots via active transport (Jones & Darrah, 1994a). (2) No active uptake mechanism has been shown to occur in roots for organic acids, and organic acids can be preferentially released under certain environmental conditions (Jones, 1998).

In vitro growth by *Ent. cloacae* strains on compounds detected in cucumber root exudate

The relative growth was determined of *Ent. cloacae* strain M2 on reduced carbon compounds detected in cucumber exudate at concentrations greater than 1 μ g per plant (Table 2) and which also supported growth by strain 501R3. The amino acids aspartate, glutamine, proline and serine; the organic acids citrate, malate and succinate; and the carbohydrates arabinose, cellobiose, galactose, glucose, mannose, rhamnose, sucrose and xylose were supplied individually in M56 salts broth at 50 mM final concentration. Strain M2, containing a mutation in *sdhA*, was substantially slower in growth relative to strain 501R3 on all amino acids and organic acids tested, and slightly slower in growth on carbohydrates when these compounds were supplied individually (data not shown). Consistent with this, strain M2 was substantially reduced in growth relative to strain 501R3 on the mixture of all amino acids and organic acids detected in cucumber root exudate (SRE-AA/OA medium) when this mixture was supplied at 0.5 (Fig. 2a), 0.1, 0.05 and 0.01 % (data not shown). It should be noted that significant growth by strain M2 was achieved by 23 h in these experiments on SRE-AA/OA medium (data not shown). Growth on the mixture of all carbohydrates detected in cucumber root exudate (SRE-CHO medium) by strains M2 and 501R3 was similar when this mixture was supplied at 0.5 (Fig. 2b), 0.1, 0.05 and 0.01 % (data not shown). Similar results were obtained in repeated experiments (data not shown).

Strain A-11, containing a mutation in *pfkA*, was substantially slower in growth relative to strain 501R3 on all carbohydrates tested, with the exception of rhamnose, but essentially unaffected in growth on amino acids and organic acids when these compounds were supplied individually (data not shown). Consistent with this, strain A-11 was substantially reduced in growth relative to strain 501R3 on the mixture of all carbohydrates detected in cucumber root exudate (SRE-CHO medium) when this mixture was supplied at 0.5 (Fig. 2b), 0.1, 0.05 and 0.01 % (data not shown). It should be noted that significant growth by strain A-11 was achieved by 23 h in these experiments on SRE-CHO medium (data not shown). Growth by strains A-11 and 501R3 on the mixture of all amino acids and organic acids detected in cucumber root

Fig. 2. Growth of *Ent. cloacae* strains 501R3, A-11 and M2 on (a) 0.5 % SRE-AA/OA and (b) 0.5 % SRE-CHO media. The complete lists of reduced carbon components in each of these media are described in Methods. (○) Strain 501R3; (▲) strain A-11; (■) strain M2. The mean $\pm$ SD OD₅₄₀ over time from a single experiment is shown. Treatments were replicated three times.

exudate (SRE-AA/OA medium) was similar when this mixture was supplied at 0.5 (Fig. 2a), 0.1, 0.05 and 0.01 % (data not shown). Similar results were obtained in repeated experiments (data not shown).

Growth by strains 501R3, M2 and A-11 was also compared on SRE-complete medium, which represented the relative individual levels of all carbohydrates, amino acids and organic acids detected in cucumber root exudate (Table 2). The carbohydrate in SRE-complete medium had a considerable influence on the growth of *Ent. cloacae*, despite the low relative quantity of carbohydrate in the medium. Conversely, the amino acids and organic acids in the SRE-complete medium had little influence on the growth of *Ent. cloacae*, despite the high relative quantity of these compounds in the medium. *Ent. cloacae* A-11, which had wild-type growth on the mixture of amino acids and organic acids in SRE-complete medium (Fig. 2a), and failed to grow over approximately 6 h on the mixture of carbohydrate in this medium (Fig. 2b), failed to increase

significantly in optical density over ~6 h in SRE-complete medium at 0.5, 0.1 and 0.05 % (Fig. 3), despite the availability for growth of relatively large quantities of amino acids and organic acids in this medium. In contrast, wild-type strain 501R3 achieved significant growth relative to strain A-11 over this time period on 0.5, 0.1 and 0.05 % SRE-complete medium (Fig. 3). *Ent. cloacae* M2, which had wild-type growth on the mixture of carbohydrates in SRE-complete medium (Fig. 2b) and failed to grow over approximately 6 h on the mixture of amino acids and organic acids in this medium (Fig. 2a), grew at essentially wild-type rates for the initial 3.5, 2.5 and 2.5 h when this medium was supplied at concentrations of 0.5, 0.1 and 0.05 %, respectively (Fig. 3), despite the availability of only small quantities of carbohydrates for growth. After these times, growth by strain M2 relative to strain 501R3 slowed, presumably due to depletion of the carbohydrate in the SRE-complete medium. Only on 0.01 % SRE-complete medium was the growth rate of strain A-11 greater than that of M2 and similar to that of strain 501R3 (Fig. 3d). The total concentration of carbohydrate in 0.01 % SRE-complete medium was only 0.00044 %, too low a concentration of reduced carbon to support growth by *Ent. cloacae*. Similar results were obtained in repeated experiments (data not shown).

The relative impact of carbohydrates and of amino acids and organic acids in synthetic cucumber root exudate was further tested in experiments in which strain 501R3 was grown concurrently on 0.5, 0.1, 0.05 and 0.01 % SRE-complete, SRE-CHO and SRE-AA/OA media (Fig. 4). In all experiments, strain 501R3 grew more rapidly on SRE-CHO medium than on SRE-AA/OA medium at a given concentration. In addition, with increasing dilution of the three media, growth by strain 501R3 on SRE-complete medium became less like that of strain 501R3 on SRE-CHO medium and more like that of strain 501R3 on SRE-AA/OA medium (Fig. 4). Presumably this was due to the dilution of the carbohydrate mixture portion of the SRE-complete medium, which supports rapid growth by *Ent. cloacae*, to levels too low to support growth. Similar results were obtained in repeated experiments (data not shown). It should be noted that synthetic cucumber root exudate was used rather than 'authentic' exudate from cucumber roots in these *in vitro* experiments to ensure the consistency of the component reduced carbon compounds available for growth in repeated experiments. Individual reduced carbon compounds within an exudate can vary from sample to sample (Table 2; D. P. Roberts and J. S. Buyer, unpublished results).

In situ* growth on reduced carbon in cucumber rhizosphere by strains of *Ent. cloacae

Strain M2, which was diminished in growth on the amino acids and organic acids in cucumber root exudate, and strain A-11, which was diminished in growth on the carbohydrates, were both significantly reduced in competitive

Fig. 3. Growth of *Ent. cloacae* strains 501R3, A-11 and M2 on SRE-complete media supplied at (a) 0.5%, (b) 0.1%, (c) 0.05% and (d) 0.01%. The complete list of reduced carbon components in this medium is described in Methods. (○) Strain 501R3; (▲) strain A-11; (■) strain M2. The mean $\pm$ SD OD₅₄₀ over time from a single experiment is shown. Treatments were replicated three times. The y-axis scale varies from panel to panel.

cucumber root colonization relative to wild-type strain 501R3 when the strains were coapplied with strain 501R3 to cucumber seeds (Tables 3 and 4). Additionally, the impact of the lost nutritional capabilities on cucumber root colonization by *Ent. cloacae* was greatest when *Ent. cloacae* was applied to cucumber seed at lower population densities (slightly below carrying capacity). Populations of strains A-11 and M2 were 76- to 7943-fold lower than those of strain 501R3 when strains A-11 and M2 were coincubated with strain 501R3 in cucumber rhizosphere during competitive cucumber root colonization experiments performed at low population densities in potting mix (Table 3). Wild-type strain 501R3 typically increased in population around fivefold over the course of these experiments, in which $\sim 4.5 \log_{10}(\text{c.f.u.})$ per seed of each bacterial strain was coapplied. Similar results were obtained in a second experiment (data not shown). Populations of strains M2 and A-11 were significantly different ($P \leq 0.018$) but only three- to 37-fold lower than those of 501R3 when these strains were coapplied to cucumber seed above carrying capacity in experiments

conducted in potting mix (Table 4). Wild-type strain 501R3 decreased in population around 10-fold over the course of these experiments, in which $\sim 7.8 \log_{10}(\text{c.f.u.})$ per seed of each bacterial strain was coapplied. Similar results were obtained in a second experiment (data not shown).

Results from experiments in which low populations [$\sim 3.5 \log_{10}(\text{c.f.u.})$ per seed] of strains M2 and 501R3 were applied individually to cucumber seeds in potting mix were consistent with those of the competitive root colonization experiments that were performed at slightly below carrying capacity. Populations of strain M2 ranged from similar to 255-fold lower than those of strain 501R3 in two experiments, and were significantly lower than those of 501R3 ($P \leq 0.004$) at one time point in both 55-day experiments (data not shown). In equivalent experiments, populations of strain A-11 ranged from similar to 2190-fold lower than those of strain 501R3, and were significantly different ($P \leq 0.05$) at three of four time points in two 55-day experiments (data not shown). Root

Fig. 4. Growth of *Ent. cloacae* strain 501R3 on SRE-complete (○), SRE-AA/OA (▲) and SRE-CHO (■) media supplied at (a) 0.5 %, (b) 0.1 %, (c) 0.05 % and (d) 0.01 %. The complete lists of reduced carbon components in these media are described in Methods. The mean $\pm$ SD OD₅₄₀ over time from a single experiment is shown. Treatments were replicated three times. The y-axis scale varies from panel to panel.

colonization experiments in which strains M2, A-11 and 501R3 were applied individually to cucumber seeds at populations above carrying capacity [$\sim 7.5 \log_{10}(\text{c.f.u.})$ per seed] were also consistent with the competitive root colonization experiments (data not shown). Populations of strain M2 ranged from two- to fivefold lower ($P > 0.05$ at all time points) than those of strain 501R3 in three of six experiments, while populations of strain M2 ranged from five- to 110-fold lower than those of strain 501R3 ($P \leq 0.05$ at all time points) in the other three experiments. Likewise, populations of strain A-11 ranged from similar to sixfold lower ($P > 0.05$ at all time points) than those of strain 501R3 in three of four experiments, and ranged from six- to 19-fold lower than those of strain 501R3 ($P \leq 0.05$ at all time points) in the other experiment.

Consistent with the *in vitro* growth experiments with the SRE media, the impact of the loss of carbohydrates as nutrients was greater than that of the loss of amino acids and organic acids on colonization by *Ent. cloacae* in competitive cucumber root colonization experiments when

A-11 and M2 were coapplied to cucumber seeds at lower population densities (Table 3). In experiments in which strains A-11 and M2 were coapplied to seeds at low population densities, populations of strain A-11 were 41- to >3000 -fold lower than those of strain M2 at all time points. In contrast, strains A-11 and M2 were equally competitive in cucumber rhizosphere when the two strains were coapplied to cucumber seeds at populations above carrying capacity (Table 4). Similar results were obtained in repeated experiments (data not shown).

DISCUSSION

To date, three major pathways that function in carbon metabolism have been shown to be important for colonization of cucumber roots by *Ent. cloacae*. Experiments reported here clearly demonstrate roles for the Embden–Meyerhof–Parnas pathway and the TCA cycle in colonization. *Ent. cloacae* mutants A-11 and M2 were both reduced in colonization of cucumber roots (Tables 3

Table 3. Competitive colonization of cucumber rhizosphere by *Ent. cloacae* strains when applied below carrying capacity

Approximately 4.5 log₁₀(c.f.u.) per seed of each strain was applied. Plants were grown in deep pots filled with potting mix. Results of a single experiment are shown.

Day	<u>log₁₀(c.f.u.)*</u>			Pr>T†	Population difference‡
	501R3	M2	A-11		
501R3 vs M2					
13	4.48	2.60	ND§	0.017	76-fold
28	4.53	1.20	ND	<0.0001	2138-fold
41	4.89	1.91	ND	0.0002	955-fold
55	4.32	1.79	ND	0.0016	339-fold
501R3 vs A-11					
13	4.63	ND	1.70	<0.0001	851-fold
28	4.78	ND	1.80	<0.0001	955-fold
41	4.27	ND	BDT	<0.0001	NC¶
55	5.22	ND	1.32	<0.0001	7943-fold
M2 vs A-11					
13	ND	3.43	0.60	0.0007	676-fold
28	ND	3.67	0.70	0.0004	933-fold
41	ND	4.13	BDT	<0.0001	NC
55	ND	2.24	0.63	0.0499	41-fold

*log₁₀(c.f.u.) was determined by dilution-plating onto media selective for each strain. Values are the mean of six replicates and expressed as mean log₁₀(c.f.u.) per root system.

†Pr>T difference in log₁₀(c.f.u.) per root system between strains in this row.

‡Difference in population sizes between paired strains at that particular sampling time.

§ND, Not determined.

||BDT, Below detectable threshold.

¶NC, Not calculated. Value could not be calculated.

and 4). Genetic and biochemical restoration of colonization by A-11 (Roberts *et al.*, 1999) strongly suggests that the reduction in colonization by this strain in experiments reported here was solely due to insertional inactivation of *pfkA*, a key enzyme in the glycolytic Embden–Meyerhof–Parnas pathway in enteric bacteria. Genetic and biochemical data presented here indicate that the defect in colonization by strain M2 was the result of insertional inactivation of the locus that contains *sdhA* and consequently the disruption of the TCA cycle. SDH functions in the TCA cycle, catalysing the oxidation of succinate to fumarate with the concurrent reduction of ubiquinone in the cell membrane under aerobic conditions. In *E. coli*, *sdhCDAB*, *sucAB* and *sucCD* form a locus with transcription initiation and regulation primarily at the upstream *sdhC* promoter (Cunningham & Guest, 1998; Park *et al.*, 1997). A weak *sucA* promoter also initiates transcription of *sucAB* and *sucCD* (Park *et al.*, 1997). In addition to *sdhCDAB* and *sucA* having the same physical orientation in *Ent. cloacae* and *E. coli*, regulatory regions of the *Ent.*

Table 4. Competitive colonization of cucumber rhizosphere by *Ent. cloacae* strains when applied above carrying capacity

Approximately 7.8 log₁₀(c.f.u.) per seed of each strain was applied. Plants were grown in deep pots filled with potting mix. Results of a single experiment are shown.

Day	<u>log₁₀(c.f.u.)*</u>			Pr>T†	Population difference‡
	501R3	M2	A-11		
501R3 vs M2					
11	6.20	5.71	ND§	0.018	Threefold
26	6.04	5.05	ND	<0.0001	10-fold
40	6.18	5.07	ND	<0.0001	13-fold
53	6.61	5.30	ND	<0.0001	20-fold
501R3 vs A-11					
11	6.44	ND	5.77	0.0065	Fivefold
26	6.07	ND	4.75	<0.0001	20-fold
40	6.15	ND	4.80	<0.0001	22-fold
53	6.39	ND	4.82	<0.0001	37-fold
M2 vs A-11					
11	ND	5.93	6.01	0.75	1.2-fold
26	ND	5.47	5.20	0.27	Twofold
40	ND	5.61	5.67	0.85	Same
53	ND	5.67	5.87	0.42	1.6-fold

*log₁₀(c.f.u.) was determined by dilution-plating onto media selective for each strain. Values are the mean of six replicates and expressed as mean log₁₀(c.f.u.) per root system.

†Pr>T difference in log₁₀(c.f.u.) per root system between strains in this row.

‡Difference in population sizes between paired strains at that particular sampling time.

§ND, Not determined.

cloacae sdhCDABsucA cluster share significant homology with those of *E. coli*. ArcA, cAMP-CRP and Fur binding sites with high DNA sequence identity to those of *E. coli* have been identified (Lynch & Lin, 1996; Shen & Gunsalus, 1997; Zhang *et al.*, 2005). In light of these similarities, the data reported here correlate *sdhA*, and possibly the *sdhCDABsucABsucCD* locus, with colonization of cucumber roots. However, *sucCD* and *sucAB* encode succinyl coenzyme A synthetase and subunits of the α -oxoglutarate dehydrogenase complex (Keseler *et al.*, 2005), respectively, which, like SDH, function in the TCA cycle. A previous study has demonstrated a role for the pentose phosphate pathway, the third major pathway for carbon metabolism in enteric bacteria, in cucumber root colonization by *Ent. cloacae* (Lohrke *et al.*, 2002).

The impact of the mutations in *sdhA* in strain M2 and in *pfkA* in strain A-11 on cucumber root colonization by *Ent. cloacae* was due to the role of these genes in deriving energy for growth and other cellular functions from reduced carbon in cucumber root exudate, and was not due to indirect effects. Although SDH functions in the TCA cycle and the TCA cycle is an important source of intermediates

for cellular biosynthesis (Guest & Russell, 1992), the mutation in *sdhA* did not impact cucumber root colonization due to the creation of auxotrophic requirements for growth, as M2 and other *sdh* mutants are prototrophs (Herbert & Guest, 1971; Roberts *et al.*, 1996). As with strain M2, the mutation in *pfkA* in A-11 did not create auxotrophic requirements for growth, as A-11 and other *pfkA* mutants are prototrophs (Roehl & Vinopal, 1976; Roberts *et al.*, 1992). There are also no reports of SDH or phosphofructose kinase A, the *pfkA* gene product, functioning as global regulators. Phosphofructose kinase A is an allosteric enzyme that sets the pace of glycolysis (Fraenkel, 1996), and as such functions primarily in regulating the catabolism of carbohydrate. Finally, metabolic flux analyses of mutants containing individual mutations in central metabolic pathways have shown that increased flux to alternative pathways can occur with indirect effects on growth (Canonaco *et al.*, 2001; Emmerling *et al.*, 2002). However, this does not appear to be the case for strains M2 and A-11, as both strains exhibited wild-type growth *in vitro* on certain substrates (Fig. 2) and in the spermospheres of certain seeds (Roberts *et al.*, 1996, 1999).

The competitive cucumber root colonization assays presented here revealed that the loci containing *sdhA* and *pfkA* were more important for colonization when bacteria were applied at populations slightly below carrying capacity than when applied at populations above carrying capacity (Tables 3 and 4). In these experiments, strain combinations were coapplied to cucumber seeds at two population levels that differed by three orders of magnitude. Differences in populations between 501R3 and A-11 and between 501R3 and M2 were much greater in competitive colonization experiments in which the strains were coapplied to cucumber seed at lower population densities. In addition, differences in populations between strains A-11 and M2 were only detected when the lower population densities of these strains were coapplied to cucumber seed. Certain environments, such as those associated with plant surfaces, are characteristically limiting to micro-organisms in one or more nutrients (Andrews, 1992; Harder & Dijkhuizen, 1983; Paulitz, 1990; Sørensen *et al.*, 2001). Gene expression studies have documented the importance of genes involved in carbon and energy metabolism and in the uptake of reduced carbon compounds under carbon-limiting conditions (Hua *et al.*, 2004). It is not known why the importance of *pfkA* and *sdhA*, and consequently the importance of carbohydrates, amino acids and organic acids, to colonization by *Ent. cloacae* varied with population density on cucumber roots. However, if *pfkA* and *sdhA* were simply important under carbon-limiting conditions, then one would expect the impact of these mutations to be greatest at the higher population densities, at which approximately three orders of magnitude more *Ent. cloacae* cells were competing for available resources than at lower population densities. A more probable explanation is that multiple resources were limiting at the higher population

levels tested, while only carbon was limiting to growth at the lower population level. Multiple-nutrient-limited growth has been well documented in the literature (Egli & Zinn, 2003). In chemostat studies that analyse competition between microbes, in which there is more than one growth-limiting nutrient, co-existence of the competitors is more likely to occur (Gottschal, 1993). This hypothesis awaits further experimentation.

The *in vitro* (Figs 3 and 4) and *in situ* (Tables 3 and 4) experiments reported here demonstrated a substantial role for carbohydrates as nutrients for growth during colonization of cucumber roots by *Ent. cloacae*, even when these carbohydrates were not available in significant quantities. The relationship between growth rate and fitness/competitiveness may explain why carbohydrate played such a substantial role in competitive root colonization. Carbohydrate supported the most rapid growth by *Ent. cloacae*, while amino acids and organic acids supported slower growth rates (Fig. 4; Roberts *et al.*, 2000). Rapid growth by microbes is thought to lead to increased fitness (Harder & Dijkhuizen, 1983; Koch, 2001).

Our studies clearly indicate a role for carbohydrate catabolism by *Ent. cloacae* in colonization of cucumber rhizosphere, in contrast to earlier published root colonization work with plant-beneficial pseudomonads (Lugtenberg *et al.*, 1999, 2001). Those authors found that a mutant with a transposon insertion in *zwf*, which encodes a key enzyme in the glycolytic Entner–Doudoroff pathway, was unaffected in competitive colonization of tomato roots. Based on work with a mutant containing a mutation just upstream of the *mdh* operon, they suggest that organic acids are the most important for colonization of tomato rhizosphere by pseudomonads. It is possible that these differences are due to differences in experimental design: the use of non-sterile planting media and cucumber plants in our studies as opposed to the use of sterile planting media and tomato plants in the work by Lugtenberg and co-workers. However, it is more likely that these differences are due to differing catabolic strategies between enteric bacteria, such as *Ent. cloacae*, and pseudomonads. Enteric bacteria have been shown to have a functional Entner–Doudoroff pathway, but the preferred catabolic route for carbohydrates in these bacteria is via the Embden–Meyerhof–Parnas pathway. The primary function of the Entner–Doudoroff pathway in enteric bacteria is thought to be in sugar acid metabolism (Peekhaus & Conway, 1998). Enteric bacteria, such as *Ent. cloacae*, clearly prefer carbohydrates to organic acids and amino acids as sources of carbon and energy, as evidenced by rapid growth on carbohydrates relative to organic acids and amino acids (Roberts *et al.*, 1992, 2000), and cAMP-CRP-mediated repression, in the presence of sugars, of enzymes that function in organic acid and amino acid catabolism (Botsford & Harman, 1992). The reverse is true for pseudomonads, in which growth rates are higher on certain organic acids (Collier *et al.*, 1996). Additionally, certain TCA cycle intermediates have been shown to

repress expression of the *hex* regulon and the glucose transporter, both of which function in the catabolism of carbohydrates by pseudomonads (Collier *et al.*, 1996). The work presented here, together with earlier work (Lugtenberg *et al.*, 1999, 2001), suggests that nutritional preferences for carbon and energy, and the importance of these nutrients, during colonization of plant roots by beneficial bacteria vary with the bacterial species.

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