Isolation of novel strains of *Agrobacterium radiobacter* with altered capacities for lactose metabolism and succinoglucon production

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Agrobacterium radiobacter NCIB 11883 was grown in continuous culture at low dilution rate under lactose limitation. Washed cells rapidly transported lactose [and its non-metabolizable analogue MTG (methyl β -D-thiogalactoside)] via a lactose-binding protein (LBP)-dependent uptake system, and subsequently hydrolysed the lactose using a highly active β -galactosidase composed of two identical subunits of M_r approximately 86000. Growth under these conditions for >40 generations led to the selection of a novel strain (AR50) which overexpressed both LBP and β -galactosidase, and exhibited lactose (MTG) uptake and β -galactosidase activities that were two to three times those of the wild-type organism. Both enzymes were expressed constitutively in strain AR50, in contrast to the wild-type organism, but remained subject to catabolite repression (particularly by galactose). Southern blotting of restricted DNA from the two organisms using an oligonucleotide probe for the structural gene for LBP showed a 2.7-fold amplification in strain AR50, together with a deletion of at least 1.7 kb which may be part of a regulatory gene. The wild-type organism and strain AR50 exhibited similar lactose (MTG) uptake rates during growth under ammonia limitation, and also synthesized an exocellular succinoglucon polysaccharide at only marginally different rates [q_p 0.21–0.24 g h⁻¹ (g cells)⁻¹]; both organisms exhibited a flux control coefficient for lactose uptake on succinoglucon production of >0.45, indicating that lactose uptake is a major kinetic control point for polysaccharide production. Growth of strain AR50 under ammonia limitation for >40 generations led to the selection of another novel strain (AR60) which exhibited a decreased q_p [0.16 g h⁻¹ (g cells)⁻¹]. Washed cells of strain AR60 exhibited significantly lower rates of lactose (MTG) uptake than strain AR50, an observation which was commensurate with the rate of polysaccharide production being predominantly controlled by the rate of lactose uptake, but β -galactosidase activity was substantially higher. Both the lactose uptake system and β -galactosidase were expressed constitutively in strain AR60, but catabolite repression of β -galactosidase was much weaker than in the wild-type organism or strain AR50.

Introduction

The aerobic bacterium *Agrobacterium radiobacter* grows on a wide range of carbon substrates, several of which are taken up via binding-protein-dependent active transport systems (for a review of binding-protein-dependent transport in bacteria, see Ames, 1986). These uptake systems are maximally expressed during growth in continuous culture under carbon limitation, and binding proteins which sequester glucose/galactose (GBP1), glucose/xylose (GBP2) and lactose (LBP) with high affinity have been purified from osmotic-shock fluids

prepared from cells grown under carbon-limited conditions (Cornish *et al.*, 1988a, 1989; Greenwood *et al.*, 1990).

Expression of the binding-protein-dependent lactose uptake system [which also transports the non-metabolizable lactose analogue methylthiogalactoside (MTG)] is closely linked to that of the β -galactosidase which subsequently hydrolyses the lactose to glucose plus galactose. Both systems are induced by lactose and other galactosides, and are subject to catabolite repression by glucose and galactose, but not by glycerol (Greenwood *et al.*, 1990).

Prolonged growth of *A. radiobacter* in continuous culture under glucose limitation has previously been used to isolate spontaneous mutants which hyperproduce either the GBP1- or GBP2-dependent uptake system (strains AR18 and AR9 respectively) and therefore

Abbreviations: GBP, glucose-binding protein; LBP, lactose-binding protein; MTG, methyl β -D-thiogalactoside; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; q_p , rate of succinoglucon production [g h⁻¹ (g cells)⁻¹].

exhibit substantially increased rates of glucose transport compared with the wild-type (Cornish *et al.*, 1988a). The two transport systems are differentially repressed by excess glucose during growth under ammonia limitation, as a result of which the cells exhibit different rates of glucose uptake (strain AR18 > strain AR9/wild-type). In each case the rate of glucose uptake is greater than that required merely to support growth, and the surplus glucose is converted into a succinoglucan exopolysaccharide at a rate which reflects the excess capacity of each organism for glucose uptake (strain AR18 > strain AR9/wild-type) (Cornish *et al.*, 1988b). Similar results have also been obtained with *A. tumefaciens* (Cornish *et al.*, 1989).

This paper reports the sequential isolation of two novel strains of *A. radiobacter* during growth in continuous culture on lactose, and describes the properties of these organisms in terms of their capacities for lactose metabolism and succinoglucan production.

Methods

Growth conditions. *Agrobacterium radiobacter* NCIB 11883 was grown in continuous culture ($D = 0.045 \text{ h}^{-1}$) at 30°C , pH 7.0 in a chemically defined, minimal salts medium (Linton *et al.*, 1987) supplemented with lactose, using an LH series 500 chemostat of 2 litre working volume as described previously (Cornish *et al.*, 1987, 1988a). The inflowing concentration of lactose was 1.9 g l^{-1} during lactose limitation, but this was increased to 18 g l^{-1} during ammonia limitation (under which conditions the concentration of ammonium sulphate was decreased from 3 to 0.5 g l^{-1}).

Batch cultures were grown into late exponential phase at 30°C , pH 7.0, in 500 ml baffled flasks containing 150 ml of minimal medium (Linton *et al.*, 1987) supplemented with lactose or other carbon sources (5 g l^{-1}).

Plate cultures were grown at 30°C for 48–64 h on minimal salts medium solidified with 1.5% (w/v) agar supplemented with various carbon sources (5 g l^{-1}) and IPTG (1 mM).

Preparation of cell suspensions and broken cells. Cells were harvested by centrifugation in an MSE High-Speed 18 centrifuge at 10000 g for 15 min, then washed in 20 mM-HEPES/KOH buffer, pH 7.0, and resuspended in the same buffer to a density of approximately $10 \text{ mg dry wt ml}^{-1}$ (Cornish *et al.*, 1987). Broken cells were prepared by disrupting washed cell suspensions using an MSE sonicator ($4 \times 30 \text{ s}$ at full amplitude, with cooling on ice).

Measurement of sugar uptake. Rates of lactose and MTG uptake were determined by measuring the incorporation of $250 \mu\text{M}$ -[D-glucose-1- ^{14}C]lactose [$0.4 \text{ mCi mmol}^{-1}$ (15 MBq mmol^{-1}); $0.1 \mu\text{Ci}$ (3.7 kBq)] and [methyl- ^{14}C]MTG [$0.5 \text{ mCi mmol}^{-1}$ (19 MBq mmol^{-1}); $0.125 \mu\text{Ci}$ (4.6 kBq)] into washed cells ($1 \text{ mg dry weight ml}^{-1}$) at 15 s intervals over a period of 1 min (Greenwood *et al.*, 1990). The procedure was as described previously for glucose uptake (Cornish *et al.*, 1988a) except that the washed filters were immediately immersed in Optiphase T scintillant (LKB) prior to counting.

Enzyme assays. The following enzymes were assayed in broken cells at 30°C using modifications of established procedures (given in parentheses): hexokinase and β -galactosidase (Bergmeyer, 1974),

glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (NADP $^{+}$ -linked) (Beardmore *et al.*, 1982), and galactose dehydrogenase (NAD $^{+}$ -linked) (Maxwell *et al.*, 1962).

MNNG mutagenesis. Cells were grown in batch culture in lactose-minimal-salts medium to a density of approximately $0.5 \text{ mg dry weight ml}^{-1}$, then harvested and resuspended in 0.1 M -citric acid/ Na_3PO_4 buffer, pH 5.5. Cells were incubated with various concentrations of MNNG ($0.5 \text{ mg cells ml}^{-1}$, 0.1 – $1.0 \text{ mg MNNG ml}^{-1}$; final volume 1.0 ml) for 15 min at 30°C , then harvested and washed twice in 0.1 M -citric acid/ Na_3PO_4 buffer, pH 5.5, and resuspended in $1.0 \text{ ml } 20\%$ (v/v) glycerol. Samples (0.1 ml) were taken from each preparation, diluted appropriately and plated on to lactose-minimal-salts agar.

Screening for mutants derepressed in lactose transport. Following mutagenesis with MNNG, cells were grown on galactose-minimal-salts agar plates for 48 h to give approximately 200 colonies per plate. The colonies were replica-plated on to galactose-minimal-salts agar plates supplemented with IPTG and overlain with an 8 cm diam. nitrocellulose disc, then incubated for approximately 8 h to allow the growth of suitably sized colonies. The disc was then removed, submerged for 15 s at room temperature in 20 mM -HEPES/KOH buffer, pH 7.0, containing $250 \mu\text{M}$ -[^{14}C]MTG of high specific activity, washed for 10 s in buffer, dried and autoradiographed.

Preparation and restriction of chromosomal DNA. Chromosomal DNA was prepared from *A. radiobacter* essentially by the method of Chow *et al.* (1977) except that the lysate was digested overnight with proteinase K before being extracted three times with phenol/chloroform and precipitated with 100% ethanol. Samples of the DNA ($5 \mu\text{g}$) were digested overnight at 37°C in a mixture containing 20 units of the restriction enzymes *Bam*HI, *Eco*RI or *Hind*III in the appropriate React buffer (BRL). The resultant restriction fragments, together with samples of chromosomal DNA, were separated by electrophoresis at 100 V for 2.5 h in 1% (w/v) agarose.

Synthesis and labelling of an oligonucleotide probe for the lactose-binding protein structural gene. A mixture of twelve 17-mer oligonucleotide probes based on a sequence of six amino acid residues (–IWCWDP–) in the N-terminal region of the LBP (Greenwood *et al.*, 1990) was synthesized via standard procedures using an Applied Biosystems 380B synthesizer, then labelled with ^{32}P at the 5'-end using T4 polynucleotide kinase (Maniatis *et al.*, 1982). Incorporation was monitored by chromatography of a small amount of the probe on DE81 paper with 0.3 M -ammonium formate as the solvent, followed by autoradiography of the dried chromatogram.

Southern blotting and hybridization. Southern blotting and hybridization were done at 37°C using standard procedures (Maniatis *et al.*, 1982) except that Hybond-N membrane was used instead of nitrocellulose during the blotting, and the hybridization buffer contained 0.25 g Marvel dried milk powder, 3 g polyethylene glycol 6000, $1.5 \times \text{SSPE}$ buffer ($1 \times \text{SSPE}$ is 0.15 M -NaCl, 10 mM - Na_2HPO_4 , 1 mM -EDTA) pH 7.4 and 1% (w/v) SDS in a final volume of 50 ml . Following hybridization, membranes were washed three times in $3 \times \text{SSC}$ buffer containing 0.1% (w/v) SDS, and then three times in $0.5 \times \text{SSC}$ buffer ($1 \times \text{SSC}$ is 0.15 M -NaCl, 15 mM -trisodium citrate) pH 7.0 containing 0.1% (w/v) SDS (all at 40°C). Washed membranes were wrapped in Saran Wrap and autoradiographed for 48 h at -70°C .

Other methods. Purification of LBP, preparation of antiserum to LBP, native (non-dissociating) PAGE, SDS-PAGE and Western blotting were done as described previously (Cornish *et al.*, 1988a, b, 1989; Silman *et al.*, 1989; Greenwood *et al.*, 1990).

Presentation of data. Where appropriate, data are given as the mean $\pm$ standard deviation with the number of independent determinations in parentheses.

Chemicals. D-[glucose-1- 14 C]Lactose [57 mCi mmol $^{-1}$ (2.11 GBq mmol $^{-1}$)] and [methyl- 14 C]MTG [50 mCi mmol $^{-1}$ (1.85 GBq mmol $^{-1}$)] were purchased from Amersham and New England Nuclear respectively. Unlabelled MTG was obtained from Sigma. Restriction enzymes and T4 ligase were obtained from BRL. All other reagents were obtained from Fisons and were of the highest grade available.

Results

Growth of A. radiobacter in continuous culture under lactose and ammonia limitation ($D = 0.045\text{ h}^{-1}$)

Washed cells of *A. radiobacter* prepared from a lactose-limited culture readily transported radiolabelled lactose, MTG and glucose (Table 1). Broken cells exhibited substantial β -galactosidase, hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and galactose dehydrogenase activities, indicating that the glucose and galactose produced by hydrolysis of the transported lactose were metabolized via the Entner-Doudoroff and de Ley-Leloir pathways respectively (Greenwood *et al.*, 1990). All of these enzyme activities were sufficient to support the rate of lactose metabolism *in vivo* by the growing organism [$> 30\text{ nmol min}^{-1}(\text{mg cells})^{-1}$ cf. approximately $5\text{ nmol min}^{-1}(\text{mg cells})^{-1}$]. SDS-PAGE of cell proteins showed the presence of a lactose-binding protein (LBP; M_r 41 000), two glucose-binding proteins (GBP1 and GBP2; M_r 36 500 and 33 500 respectively) and an as yet unidentified putative binding protein (BP3; M_r 30 500) which ranged in concentration from < 2 to 18% of the total cell protein as determined by scanning densitometry (Fig. 1; Table 1; see also Greenwood *et al.*, 1990). Native PAGE of broken cells followed by exposure to X-Gal allowed β -galactosidase to be detected via the formation of the blue hydrolysis product; the enzyme was subsequently electroeluted from an excised, unstained band and subjected to SDS-PAGE which revealed a single subunit of M_r 86 000 and indicated that the enzyme probably constituted $< 2\%$ of the total cell protein. Gel-filtration of a high-speed supernatant fraction prepared from sonicated cells gave a native M_r of 153 000 for β -galactosidase, indicating that it existed as a homodimer (α_2).

When the culture was switched to ammonia limitation the lactose (MTG) transport and β -galactosidase activities were decreased by approximately three-quarters, whereas the glucose transport, hexokinase and 6-phosphogluconate dehydrogenase activities were variably decreased by up to one-half, and the glucose-6-phosphate dehydrogenase and galactose dehydrogenase activities were slightly increased. SDS-PAGE showed that the concentrations of LBP, GBP1 and BP3 were also substantially decreased [LBP and GBP1 approximately in parallel with the decrease in the rates of lactose

Fig. 1. Production of LBP and other binding proteins by *A. radiobacter* wild-type and strain AR50 during growth in continuous culture under lactose and ammonia limitation ($D = 0.045\text{ h}^{-1}$). Cellular proteins were separated using SDS-PAGE and either stained for protein with Kenacid blue (a) or transferred to nitrocellulose and probed with antiserum raised against LBP (b) as described in Methods. Tracks: 1, M_r standards; 2, lactose-limited wild-type; 3, ammonia-limited wild-type; 4, lactose-limited strain AR50; 5 ammonia-limited strain AR50.

(MTG) and glucose uptake respectively] such that each binding protein constituted $< 2\%$ of the total cell protein. During growth under these excess-lactose conditions the organism synthesized a succinoglucan exopolysaccharide of identical composition to, and at the same rate [q_p $0.21\text{ g h}^{-1}(\text{g cells})^{-1}$] as that observed previously during growth on excess glucose (Cornish *et al.*, 1988b). As a result of succinoglucan synthesis, the rate of lactose metabolism *in vivo* by the ammonium-limited culture was significantly higher than that of the lactose-limited culture growing at the same dilution rate [approximately 15 cf. $5\text{ nmol min}^{-1}(\text{mg cells})^{-1}$]. This rate was easily exceeded by all of the enzyme activities measured except for the lactose (MTG) uptake system which was only 9 to $10\text{ nmol min}^{-1}(\text{mg cells})^{-1}$. If it is assumed that the latter was slightly underestimated, the results suggested that the rate at which an ammonia-limited culture of *A. radiobacter* consumed lactose was largely determined by the activity of its lactose uptake system.

Table 1. *Properties of A. radiobacter following growth in lactose-limited and ammonium-limited continuous culture ($D = 0.045 \text{ h}^{-1}$)*

Enzyme activities, binding protein concentrations and *in vivo* succinoglucan production rates (q_p) were measured as described in Methods. Values are means $\pm$ SEM, with the number of determinations in parentheses. Binding protein concentrations in cells grown under ammonia limitation were too low to be determined accurately ($\leq 2\%$).

Property	Lactose-limited	Ammonium-limited	Ratio
(a) Enzyme activity [nmol min ⁻¹ (mg cells) ⁻¹]			
Lactose uptake	35 $\pm$ 2(10)	9 $\pm$ 1(3)	0.26
MTG uptake	39 $\pm$ 1(4)	10 $\pm$ 1(3)	0.26
Glucose uptake	30 $\pm$ 1(5)	16 $\pm$ 0(3)	0.53
β -Galactosidase	271 $\pm$ 11(7)	79 $\pm$ 4(3)	0.29
Galactose dehydrogenase	39 $\pm$ 5(7)	53 $\pm$ 3(3)	1.35
Hexokinase	58 $\pm$ 1(3)	45 $\pm$ 3(3)	0.78
Glucose-6-phosphate dehydrogenase	36 $\pm$ 3(3)	40 $\pm$ 9(3)	1.11
6-Phosphogluconate dehydrogenase	66 $\pm$ 3(2)	49 $\pm$ 2(3)	0.74
(b) Binding protein concentrations (% cell protein)			
LBP	8 $\pm$ 1(6)		
GBP1	6 $\pm$ 0(6)		
GBP2	<2		
BP3	18 $\pm$ 1(6)		
(c) Succinoglucan production [g h ⁻¹ (g cells) ⁻¹]			
	0	0.21 $\pm$ 0.01(3)	

Isolation of a strain of A. radiobacter with an enhanced rate of lactose uptake following prolonged growth in continuous culture under lactose limitation

The growth of *A. radiobacter* under lactose limitation ($D = 0.045 \text{ h}^{-1}$) led after about 40 generations to a sudden, approximately 3-fold increase in the rate of lactose (MTG) uptake by washed cells. This was accompanied by a similar increase in the concentration of LBP.

A small sample of the culture was streaked on to a lactose-minimal-salts agar plate, and 24 of the resultant colonies were grown in small-scale batch cultures until the lactose was exhausted. SDS-PAGE of the washed cells showed that the chemostat culture was essentially homogeneous. A similar conclusion was also reached when the same 24 colonies were grown in batch culture into ammonia exhaustion and then examined in the same way.

One colony (subsequently called strain AR50) was picked at random and grown in lactose-limited continuous culture ($D = 0.045 \text{ h}^{-1}$). The cells exhibited lactose (MTG) uptake, β -galactosidase and glucose-6-phosphate dehydrogenase activities that were two to three times those of the wild-type organism grown under similar conditions, whereas the glucose uptake, galactose

dehydrogenase, hexokinase and 6-phosphogluconate dehydrogenase activities were essentially unchanged (Table 2). SDS-PAGE showed that the subunit M_r values of LBP and β -galactosidase were the same as in the wild-type organism. Scanning densitometry subsequently demonstrated that the concentrations of LBP and β -galactosidase were three to four times those of the wild-type organism, and that LBP constituted approximately 30% of the total cell protein (as the concentration of β -galactosidase was determined by overloading the gels and scanning only the top one-third, it was not possible to express it as a percentage of the total cell protein). In contrast, the concentrations of GBP1 and GBP2 were unchanged, and the concentration of BP3 was significantly decreased.

When grown under ammonia limitation the activities of the lactose (MTG) uptake system and all the other enzymes assayed were generally similar to those exhibited by wild-type cells, i.e. the lactose (MTG) uptake system, β -galactosidase and glucose-6-phosphate dehydrogenase which were hyperexpressed in lactose-limited AR50 were all strongly repressed during growth under ammonia limitation. The rate of succinoglucan production by strain AR50 was also only marginally different to that of the wild-type organism [0.24 cf. 0.21 g h⁻¹ (g cells)⁻¹].

Table 2. Properties of *A. radiobacter* strain AR50 following growth in lactose-limited and ammonium-limited continuous culture ($D = 0.045 \text{ h}^{-1}$)

Enzyme activities, binding protein concentrations and *in vivo* succinoglucan production rates (q_p) were measured as described in Methods. Values are means $\pm$ SEM, with the number of determinations in parentheses. Strain AR50 was selected by prolonged growth under lactose limitation as described in the text. Binding protein concentrations in cells grown under ammonia limitation were too low to be determined accurately ($\leq 2\%$).

Property	Lactose-limited	Ammonium-limited	Ratio
(a) Enzyme activity [nmol min ⁻¹ (mg cells) ⁻¹]			
Lactose uptake	76 $\pm$ 1(2)	8 $\pm$ 1(8)	0.10
MTG uptake	82 $\pm$ 1(2)	9 $\pm$ 2(3)	0.11
Glucose uptake	26 $\pm$ 2(4)	13 $\pm$ 1(4)	0.50
β -Galactosidase	625 $\pm$ 10(2)	107 $\pm$ 5(4)	0.17
Galactose dehydrogenase	44 $\pm$ 3(4)	73 $\pm$ 5(4)	1.66
Hexokinase	63 $\pm$ 11(3)	50 $\pm$ 7(4)	0.79
Glucose-6-phosphate dehydrogenase	88 $\pm$ 2(3)	33 $\pm$ 7(4)	0.38
6-Phosphogluconate dehydrogenase	62 $\pm$ 2(3)	62 $\pm$ 4(4)	1.00
(b) Binding protein concentration (% cell protein)			
LBP	30 $\pm$ 1(4)		
GBP1	5 $\pm$ 0(4)		
GBP2	<2		
BP3	12 $\pm$ 1(4)		
(c) Succinoglucan production [g h ⁻¹ (g cells) ⁻¹]			
	0	0.24 $\pm$ 0.01(7)	

Table 3. Lactose uptake and β -galactosidase activities of *A. radiobacter* wild-type, strain AR50 and strain AR60 following growth on various carbon sources

A. radiobacter wild-type, strain AR50 and strain AR60 were grown for 48 h on minimal-salts agar supplemented with various carbon sources $\pm$ IPTG. Whole cells and broken cells were prepared and assayed for lactose uptake and β -galactosidase activities respectively as described in Methods. The results are means of up to three independent determinations.

Carbon source	Lactose uptake			β -Galactosidase		
	WT	AR50	AR60	WT	AR50	AR60
	[nmol min ⁻¹ (mg cells) ⁻¹]					
Glycerol	0	34	10	0	833	1667
Glucose	0	26	19	0	854	1454
Galactose	0	7	7	20	559	1083
Lactose	8	8	6	308	417	1367
Glycerol + IPTG	4	33	16	92	800	1479
Glucose + IPTG	4	27	36	121	880	1792
Galactose + IPTG	3	10	4	83	517	1233

Induction and repression of lactose uptake and β -galactosidase during the growth of the wild-type and strain AR50 on solid medium

Previous work from this laboratory had shown that expression of lactose (MTG) uptake and β -galactosidase by wild-type *A. radiobacter* growing in batch culture was induced by lactose and other galacto-

sides (including IPTG) and was subject to catabolite repression by carbon substrates such as glucose and galactose, but not glycerol, that allowed rapid growth of the organism (Greenwood *et al.*, 1990). This pattern of induction and catabolite repression was largely confirmed for wild-type *A. radiobacter* grown on solid medium (Table 3), except that IPTG was a less-effective inducer and glycerol was a more-effective catabolite

Fig. 2. Southern analysis of DNA restriction fragments from *A. radiobacter* wild-type and strain AR50 using a ^{32}P -labelled oligonucleotide probe for the gene for LBP. DNA was prepared from the wild-type organism and strain AR50, and then digested with three different restriction endonucleases. The restriction digests were electrophoresed on an agarose gel, then transferred to a Hybond-N membrane, hybridized and autoradiographed as described in Methods. Restriction endonucleases: H, *Hind*III; E, *Eco*RI; B, *Bam*HI.

repressor than during growth in batch culture. In contrast, expression of lactose uptake and β -galactosidase by strain AR50 was essentially constitutive and generally to a much higher level than in the wild-type organism; this expression remained sensitive to catabolite repression, albeit with specificity similar to that observed for the wild-type organism growing in batch culture (galactose > glucose > glycerol).

Southern analysis of DNA restriction fragments from A. radiobacter wild-type and strain AR50

LBP from wild-type *A. radiobacter* had previously been shown to contain a sequence of six amino acids (–IWCWDP–) within the N-terminal region which was particularly suitable for constructing an oligonucleotide probe (Greenwood *et al.*, 1990). A mixture of twelve 17-mer oligonucleotides was therefore synthesized [ATT(CA) TGG TGT(C) TGG GAT(C) CC]. This was subsequently labelled at the 5'-end with ^{32}P and used to Southern blot restriction digests of DNA prepared from the wild-type organism and strain AR50. The resultant autoradiograph showed that the probe hybridized strongly with a single fragment in each digest of the wild-type DNA, viz. an approximately 26 kb *Bam*HI fragment, a 14.2 kb *Hind*III fragment and a 5.5 kb *Eco*RI fragment (Fig. 2). It also hybridized strongly with a single fragment in each of the restriction digests of DNA prepared from strain AR50, but each of these fragments was at least 1.7 kb smaller. Scanning densitometry of the autoradiograph showed that the gene coding for LBP had on average been amplified 2.7-fold in strain AR50 compared with the wild-type, thus explaining the hyperexpression of LBP during growth under lactose limitation. As this amplification was also similar to the

observed increases in the lactose (MTG) uptake rate, β -galactosidase activity and β -galactosidase concentration (2.2 to 3.8-fold) in strain AR50, it is possible (but not proven) that all of the genes coding for these enzymes had been amplified concomitantly. Control experiments in which an oligonucleotide probe for GBP1 was used to Southern blot restriction digests of wild-type and AR50 DNA showed that no amplification of the GBP1 gene had occurred, exactly as expected from the similar GBP1 concentrations and glucose uptake rates of the two strains.

The decreased size of the hybridizing fragments from AR50 DNA compared with wild-type DNA indicated that amplification had also been accompanied by a substantial deletion of DNA. As lactose uptake and β -galactosidase activities were expressed constitutively in strain AR50, but were inducible in the wild-type organism, it is possible that the deletion included at least part of a regulatory gene.

Flux control analysis of the wild-type organism and strain AR50

Flux control analysis of washed cells of *A. radiobacter* wild-type and strain AR50 was done as described previously (Cornish *et al.*, 1988b) except that [^{14}C]glucose was replaced by [^{14}C]lactose (1 mM) and unlabelled MTG (5–40 mM) was used to inhibit [^{14}C]lactose utilization and [^{14}C]succinoglucan production. In both organisms the rates of lactose utilization and succinoglucan production were strongly inhibited by MTG, indicating a flux control coefficient of > 0.45 for lactose uptake on succinoglucan production. This value indicated that lactose uptake was a major kinetic control point for succinoglucan production in both organisms, as also was glucose uptake on succinoglucan production by strain AR18 (Cornish *et al.*, 1989b). It was concluded, therefore, that the key to enhancing the rate of succinoglucan production from lactose was to make the lactose (MTG) uptake system less susceptible to catabolite repression by the glucose and galactose produced by lactose hydrolysis, such that the activity of the uptake system is significantly increased during growth under ammonia limitation. In view of the much higher potential for lactose (MTG) uptake in strain AR50 compared with the wild-type organism, attempts were made to isolate catabolite-derepressed mutants of strain AR50.

Attempts to isolate mutants of strain AR50 derepressed for lactose (MTG) uptake

In order to detect mutants of *A. radiobacter* derepressed for lactose uptake it was necessary to develop a suitable

screening procedure, preferably a simple plate-assay based on the ability to detect lactose (MTG) uptake and/or β -galactosidase activities of single colonies. *A. radiobacter* wild-type and strain AR50 were therefore grown on solid medium containing glycerol or galactose ($\pm$ IPTG) in order to produce colonies with differing lactose (MTG) uptake and β -galactosidase activities, and attempts were made to detect these differences using either [^{14}C]MTG (to detect the activity of the lactose uptake system) or X-Gal (to detect β -galactosidase activity). The use of X-Gal was unsuccessful since formation of the blue hydrolysis product was only quantitative at low β -galactosidase activities. In contrast, autoradiograms of colonies which had been exposed to [^{14}C]MTG for 15 s showed that the amount of radioactivity present in the colonies reflected the MTG (lactose) uptake rates of washed cells measured in parallel experiments.

A. radiobacter strain AR50 was mutagenized with MNNG, then grown on minimal-salts agar containing IPTG + galactose, and subsequently screened for derepression of the lactose uptake system using [^{14}C]MTG. Over 6000 colonies were screened in this way, but no evidence was obtained for the presence of a derepressed mutant.

Decay of succinoglucan production by strain AR50 during prolonged growth under ammonia limitation

The propensity of *A. radiobacter* strain AR50 to degenerate with respect to succinoglucan production during prolonged growth was determined by growing the organism on lactose in ammonia-limited continuous culture ($D = 0.045 \text{ h}^{-1}$) for up to 40 d (62 generations), and measuring the rate of succinoglucan production at regular intervals. The q_p remained at $0.25 \pm 0.02 \text{ g h}^{-1} (\text{g cells})^{-1}$ for about 20 generations, then dropped rapidly to approximately $0.16 \text{ g h}^{-1} (\text{g cells})^{-1}$ over the next 20 generations and remained at this value for the rest of the experiment. The q_p of strain AR50 growing on lactose was therefore significantly more resistant to long-term decay than that of strain AR9 growing on glucose (Cornish *et al.*, 1988b). Colony analysis of the decayed culture of strain AR50 indicated that it was essentially homogeneous, and one colony (subsequently called strain AR60) was studied in more detail.

Properties of A. radiobacter strain AR60 during growth under ammonia and lactose limitation in continuous culture

Strain AR60 grown on lactose in ammonia-limited continuous culture ($D = 0.045 \text{ h}^{-1}$) exhibited a q_p of

$0.16 \text{ g h}^{-1} (\text{g cells})^{-1}$, identical to that of the decayed culture of strain AR50 and only two-thirds that of the undecayed culture (Table 4). The lactose and MTG uptake rates of strain AR60 were also respectively one-half and three-quarters those of undecayed strain AR50, an observation which was commensurate with the uptake system remaining a major kinetic control point for succinoglucan production. In contrast, and rather unexpectedly, β -galactosidase activity was substantially higher in strain AR60 than in undecayed strain AR50. The activities of all of the other enzymes assayed were generally similar to those exhibited by undecayed strain AR50 (and the wild-type organism) during growth under ammonia limitation, except for glucose-6-phosphate dehydrogenase activity which was substantially lower.

When strain AR60 was grown under lactose limitation, the lactose (MTG) uptake rates were similar to those of undecayed strain AR50 grown under the same conditions, but the concentration of LBP was slightly higher (again at the further expense of the BP3 concentration) suggesting that the binding protein may have been marginally overexpressed relative to the rest of the uptake system. In contrast, the β -galactosidase activity of strain AR60 was over three times higher than that of undecayed strain AR50 (and over eight times higher than that of the wild-type organism grown under the same conditions), but all the other enzyme activities remained essentially unchanged.

Native PAGE followed by activity staining for β -galactosidase using X-Gal showed that the AR60 enzyme ran with an identical R_F value to that exhibited by the enzyme in the undecayed strain AR50 and the wild-type organism, and also confirmed that strain AR60 exhibited a significantly higher β -galactosidase activity. SDS-PAGE of the excised AR60 β -galactosidase, together with gel-filtration FPLC of a high-speed supernatant fraction prepared from broken cells, revealed no changes in the subunit or native M_r of the enzyme. SDS-PAGE and scanning densitometry of overloaded broken-cell samples showed that the increased β -galactosidase activity of strain AR60 was accompanied by a similar 3-fold increase in the concentration of the enzyme, which was thus present at approximately nine times the concentration of the enzyme in the wild-type organism.

Induction and repression of lactose uptake and β -galactosidase during the growth of strain AR60 on solid medium

The growth of *A. radiobacter* strain AR60 on solid medium containing various carbon sources $\pm$ IPTG showed that the expression of the lactose uptake system in strain AR60 was still constitutive (although probably

Table 4. *Properties of A. radiobacter strain AR60 following growth in lactose-limited and ammonium-limited continuous culture (D = 0.045 h⁻¹)*

Enzyme activities, binding protein concentrations and *in vivo* succinoglucan production rates (q_p) were measured as described in Methods. Values are means $\pm$ SEM, with the number of determinations in parentheses. ND, Not determined. Binding protein concentrations in cells grown under ammonia limitation were too low to be determined accurately ($\leq 2\%$).

Property	Lactose-limited	Ammonium-limited	Ratio
(a) Enzyme activity [nmol min ⁻¹ (mg cells) ⁻¹]			
Lactose uptake	75 $\pm$ 3(6)	4 $\pm$ 0(6)	0.05
MTG uptake	67 $\pm$ 2(6)	7 $\pm$ 1(6)	0.10
Glucose uptake	ND	ND	
β -Galactosidase	2247 $\pm$ 63(5)	160 $\pm$ 7(4)	0.07
Galactose dehydrogenase	24 $\pm$ 2(7)	41 $\pm$ 2(4)	1.71
Hexokinase	35 $\pm$ 1(5)	45 $\pm$ 7(4)	1.29
Glucose-6-phosphate dehydrogenase	100 $\pm$ 5(3)	8 $\pm$ 1(4)	0.08
6-Phosphogluconate dehydrogenase	51 $\pm$ 1(5)	68 $\pm$ 5(4)	1.33
(b) Binding protein concentration (% cell protein)			
LBP	38 $\pm$ 1(4)		
GBP1	6 $\pm$ 0(4)		
GBP2	3 $\pm$ 0(4)		
BP3	5 $\pm$ 0(4)		
(c) Succinoglucan production [g h ⁻¹ (g cells) ⁻¹]			
	0	0.16 $\pm$ 0.01(4)	

less so than in strain AR50 grown under the same conditions) and also remained subject to catabolite repression, albeit with an altered substrate specificity (galactose > glycerol > glucose) compared with strain AR50 (Table 3). β -Galactosidase was also expressed constitutively in strain AR60, but at a very high level compared with strain AR50, and it was also significantly less sensitive to catabolite repression.

Discussion

The results described in this paper show that prolonged growth of *A. radiobacter* at low dilution rate in lactose-limited continuous culture can be used to select for a spontaneous mutant (strain AR50) which hyperproduces the LBP-dependent lactose transport system. In this respect, strain AR50 is analogous to strains AR18 and AR9 which hyperproduce the GBP1- and GBP2-dependent glucose transport systems respectively, and which were selected by prolonged growth of *A. radiobacter* under glucose limitation (Cornish *et al.*, 1988a). However, these three strains differ in that in strain AR50 the activity of the next enzyme in the pathway of lactose metabolism (β -galactosidase) is increased virtually in parallel with that of the lactose transport system, whereas in strains AR18 and AR9 the activity of the next

enzyme in the pathway of glucose metabolism (hexokinase) remains similar to that of the wild-type organism. Although there is good evidence from the Southern blotting experiments that hyperproduction of the lactose transport system in strain AR50 is due to gene amplification, it remains to be determined whether this is also true for the glucose transport systems in strains AR9 and AR18 (or indeed for β -galactosidase in strain AR50).

Mutants of *Escherichia coli* which hyperproduce both the lactose transport system and β -galactosidase have also been isolated using lactose-limited continuous culture (see Dykhuizen & Hartl, 1983; Dykhuizen *et al.*, 1987). In this organism, however, lactose uptake occurs via the classical Lac permease (a lactose-H⁺ symport system composed of a single transmembrane protein), and the genes coding for the permease (*lacY*) and the β -galactosidase (*lacZ*) are known to comprise part of the *lac* operon.

In contrast to the much greater capacity of *A. radiobacter* strain AR50 for lactose uptake and hydrolysis compared with the wild-type organism during growth under lactose limitation, both of these enzymes are very strongly repressed during growth under ammonia limitation with the result that the two organisms exhibit similar rates of lactose uptake and hydrolysis when grown under these conditions. They also synthesize succinoglucan at

similar rates, an observation which is entirely commensurate with the flux control analysis which showed that lactose transport is a major kinetic control point for succinoglucan production in both the wild-type organism and strain AR50. It would appear, therefore, that the key to synthesizing succinoglucan at a faster rate is to derepress the sugar uptake system during growth under ammonia limitation (as has previously been shown for the production of succinoglucan from glucose by strain AR18; see Cornish *et al.*, 1988*b*, 1989). Unfortunately, the lactose uptake system of strain AR50 resembles the glucose uptake system of strain AR9 in being strongly repressed during growth under ammonia limitation, and attempts to isolate mutants of strain AR50 which are derepressed for lactose uptake were unsuccessful.

In contrast to the effect of prolonged growth under lactose limitation, which leads to hyperproduction of the lactose uptake system (strain AR50), prolonged growth under ammonia limitation causes a significant decrease in the rate of lactose uptake (strain AR60). This is accompanied by an approximately commensurate decrease in the rate of succinoglucan production, indicating that lactose uptake is still a major kinetic control point for the formation of this product. Unexpectedly, however, strain AR60 exhibits a markedly increased β -galactosidase activity which clearly shows that lactose uptake and hydrolysis are not regulated coordinately.

It is now generally accepted that novel strains emerge under strongly selective conditions, such as growth in continuous culture, because they undergo mutations which endow them with an increased 'biological fitness' for that particular environment (Dykhuizen *et al.*, 1987). When various sugars are limiting for growth, this increased fitness is most easily acquired by hyperproducing the relevant uptake system (see also Dykhuizen *et al.*, 1987; Cornish *et al.*, 1988*a*, 1989). Similarly, when growth is limited by the availability of ammonia or other anabolic substrates it might be expected that a novel strain would emerge with an enhanced capacity for the uptake and/or assimilation of that substrate. However, since the growth of *A. radiobacter* under these conditions is accompanied by the synthesis of large amounts of succinoglucan, it is possible that other mutations would also be of benefit to the organism. Assuming that succinoglucan serves only as a means of disposing of excess sugar taken into the cell (Cornish *et al.*, 1988*a*, *b*), increased fitness might be achieved by decreasing the flux of carbon through the cell towards that required simply for the production of cell material (i.e. by decreasing the rate of succinoglucan production). It is now clear that this occurs not only during the growth of strain AR9 on glucose (Cornish *et al.*, 1988*b*), but also during the growth of strain AR50 on lactose. Whether this decreased rate of succinoglucan production stems

from a lowered capacity for sugar uptake (which leads directly to a decrease in the rate of succinoglucan production) or from a lowered capacity for polysaccharide synthesis (which leads to enhanced repression of the sugar uptake system) remains to be determined. It is perhaps pertinent to note, however, that the extent to which the rate of succinoglucan production from glucose decreases in strain AR9 is much greater than from lactose in strain AR50 over a comparable time period. Since the two strains obviously use different uptake systems, but probably synthesize succinoglucan from precursors using a common pathway, it is likely that the primary cause of the decrease in the rate of succinoglucan production is a progressive loss of uptake capacity rather than of biosynthetic capacity.

The increased capacity for lactose hydrolysis (increased β -galactosidase activity) which strain AR50 gains over this same period is presumably also of benefit to the cell, possibly by compensating for the decreased capacity for lactose uptake.

The opposing responses of the lactose uptake system and β -galactosidase during the prolonged growth of strain AR50 under ammonia limitation (and subsequently in strain AR60 isolated from this culture), together with the results obtained following the growth of the wild-type and various mutant strains on solid medium supplemented with various carbon sources, support our previous conclusion (Greenwood *et al.*, 1990) that these two enzymes are not expressed coordinately, i.e. their genes do not comprise a single operon analogous to that of the *lac* operon in *E. coli*.

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