Biochemical and genetic analysis of *Streptococcus mutans* α -galactosidase

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The *aga* gene coding for α -galactosidase in *Streptococcus mutans* was detected in a recombinant gene library constructed in phage λ . The gene was subcloned into plasmid vectors and shown to specify a novel protein of M_r 80000. Characterization of α -galactosidase from *S. mutans* and from recombinant *Escherichia coli* expressing *aga* indicated that the enzyme functions as a tetramer. The amino acid composition of the α -galactosidase, deduced from nucleotide sequencing of *aga*, gave a predicted M_r of 82022 and revealed regions of homology to α -galactosidases encoded by the *E. coli* Raf plasmids and by *Bacillus stearothermophilus*. Inactivation of the *aga* gene in *S. mutans* resulted in loss of all α -galactosidase activity and abolished the ability to ferment melibiose; α -glucosidase activity was also lost, due to an indirect effect on the *dexB* gene.

Introduction

The oral bacterium *Streptococcus mutans* is believed to be the major causative agent of dental caries in humans, a disease in which the tooth enamel is attacked by acid end-products of bacterial metabolism of fermentable carbohydrates (Hamada & Slade, 1980; Loesche, 1986). Much attention has been focused upon the utilization of sucrose, which can be utilized to form extracellular polymers important in dental plaque formation as well as being used as a fermentable carbon source. However, *S. mutans* is capable of producing acid from a wide range of carbohydrates, which may be derived from the diet or from degradation of host glycoproteins, so the pathways by which these are metabolized are also of interest in explaining the survival and multiplication of *S. mutans* in the oral cavity and the processes leading to dental caries.

We have previously described the cloning and sequencing of the closely-linked *S. mutans* genes *gtfA* and *dexB*. These encode the intracellular enzymes sucrose phosphorylase and dextran glucosidase respectively (Ferretti *et al.*, 1988; Russell & Ferretti, 1990; Russell *et*

al., 1988). We have now identified a further gene associated with carbohydrate metabolism in the same region of the chromosome which encodes α -galactosidase (α -D-galactoside galactohydrolase, EC 3.2.1.22), the gene being named *aga*.

Methods

Bacteria, vectors and growth media. *S. mutans* strains were grown in Brain Heart Infusion broth (Oxoid) or the medium described by Terleckyj *et al.* (1975) with 0.5% (w/v) casein hydrolysate instead of the individual amino acids. Carbohydrates (0.5%, w/v) or erythromycin (10 μ g ml⁻¹) were added as required. Conditions for growth and handling of *Escherichia coli* strain JM109, plasmids pUC18 and pUC19 and the single-stranded bacteriophage vectors M13mp18 and M13mp19 were as described by Yanisch-Perron *et al.* (1985). *E. coli* strain M2508 (*melA meta lac*) was kindly provided by Dr R. Schmitt (Schmitt, 1968). Plasmid pVA891 was kindly provided by Dr F. Macrina (Macrina *et al.*, 1983). *E. coli* strains were grown with shaking in Luria Broth (Maniatis *et al.*, 1982) with antibiotics as required: ampicillin (100 μ g ml⁻¹), erythromycin (400 μ g ml⁻¹).

Reagents. Isopropyl β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X- β -Gal) were obtained from Northumbria Biologicals. 5-Bromo-4-chloro-3-indolyl α -D-galactopyranoside (X- α -Gal) and calf intestinal alkaline phosphatase were from Boehringer Mannheim. T4 DNA ligase was obtained from Pharmacia. Restriction endonucleases were purchased from Northumbria Biologicals and Bethesda Research Laboratories and used according to the manufacturers' instructions. All other reagents and

Abbreviation: α -PNP-Gal, α -p-nitrophenyl galactoside.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M6077.

chemicals were of the highest purity available, and were purchased from Sigma or BDH Chemicals.

Initial cloning of *aga*. As described previously (Russell *et al.*, 1985) a gene library of random fragments of *S. mutans* strain Ingbritt chromosomal DNA, constructed in bacteriophage λ L47.1, was screened on minimal medium containing sucrose or raffinose as carbon source for the presence of recombinant plaques which released monosaccharides and so cross-fed the surrounding *E. coli*.

Nucleotide sequencing. From λ IS42, a recombinant of bacteriophage λ L47.1 previously shown to carry an insert of chromosomal DNA from *S. mutans* strain Ingbritt including the *gtfA* gene (Russell *et al.*, 1985) a series of *EcoRI* fragments were isolated. In the work leading to this report, the 4.48 kb fragment was unidirectionally digested with *Bal31* and a series of nested deletion derivatives subcloned into bacteriophage M13 vectors mp18 and mp19 for sequencing by the dideoxy chain-termination method as previously described (Ferretti *et al.*, 1986). All sequences were confirmed from at least two overlapping clones, and the entire sequence was determined on both strands. The sequence information was analysed by the James M. Pustell DNA/Protein sequencing program (International Biotechnologies) and Staden-Plus programs (Amersham). Comparison with protein sequences held in the Protein Information Resource of the National Biological Research Foundation database was by the FASTP program of Wilbur & Lipman (1982).

Subcloning of the *aga* gene. Inspection of the distribution of restriction sites and coding regions within the nucleotide sequence indicated that the entire open reading frame suspected of carrying *aga* was contained in a 2.7 kb *AvaII* fragment. Such a fragment was therefore isolated from the original recombinant phage λ IS42 by preparative agarose electrophoresis, the 'sticky ends' filled by use of Klenow fragment of DNA polymerase and the fragment ligated into the *SmaI* sites of plasmid vectors pUC18 and pUC19 to generate recombinant plasmids pSF110 and pSF111. Plasmid pSF106 was constructed by ligating the 4.48 kb *EcoRI* fragment carrying *aga* and flanking regions into pUC18.

Agarose gel electrophoresis, electro-elution, dephosphorylation, ligation of DNA and transformation of *E. coli* cells were as described by Maniatis *et al.* (1982). Plasmid DNA was routinely prepared using Qiagen Tips (Hybaid, London, UK).

Gene inactivation. Plasmid pSF110 was digested to completion with the restriction enzyme *HindIII* and fractionated by electrophoresis through a 1.2% (w/v) agarose gel in TBE buffer (90 mM-Tris, 90 mM-boric acid, 2 mM-EDTA). A 0.45 kb internal fragment of the *aga* gene was excised from the gel, electro-eluted, and purified by sequential extractions with phenol and chloroform. The fragment was ligated with T4 ligase to the plasmid pVA891 (Macrina *et al.*, 1983) which had been linearized with *HindIII* and dephosphorylated with alkaline phosphatase, to form plasmid pSF122. Plasmid pSF122 was used to transform *S. mutans* LT11 to erythromycin resistance, essentially as described by Perry & Kuramitsu (1981). *S. mutans* LT11 is a highly-transformable strain to be described in detail elsewhere (L. Tao, unpublished). The recombinant strain of *S. mutans* in which *aga* had been inactivated was named strain 516.

To confirm that plasmid pSF122 had been inserted into *aga*, chromosomal DNA from *S. mutans* strains Ingbritt, LT11 and 516 was isolated and digested to completion with the restriction enzyme *EcoRI*. After agarose gel electrophoresis, the DNA was transferred to Hybond N⁺ membrane as described below and probed with ³²P-labelled 4.48 kb *EcoRI*-insert from plasmid pSF106.

Hybridization of DNA. Chromosomal DNA (2–5 μ g) was digested completely with the restriction endonuclease *EcoRI*, electrophoresed through a 0.8% (w/v) agarose gel, and after pretreatment (Maniatis *et al.*, 1982) the DNA fragments were transferred onto Hybond N⁺ nylon

filters (Amersham) by means of a vacuum blotting apparatus (Hybaid) and pre-hybridized in 5 $\times$ SSC (Maniatis *et al.*, 1982) containing 1% (w/v) Blocking Reagent (Boehringer Mannheim), 0.1% (w/v) sodium *N*-lauroylsarcosine and 0.02% (w/v) SDS at 65 °C. The immobilized DNA was probed with the 4.48 kb *EcoRI* restriction fragment of plasmid pSF106, which had been labelled with ³²P by the random-primer method (Du-Pont Chemicals). Filters were washed with 2 $\times$ SSC plus 0.2% (w/v) SDS for 2 $\times$ 15 min and then with 0.2 $\times$ SSC plus 0.2% (w/v) SDS for 2 $\times$ 15 min. Autoradiograms were prepared using Fuji or Kodak X-OMAT-AR films at –70 °C for 2–16 h.

Fermentation tests. The ability of wild-type and mutant strains of *S. mutans* to ferment carbohydrates was tested by inoculating wells of a microtitre tray containing Purple Broth Base (16 g l^{–1}, Difco) and Thioglycollate medium (24 g l^{–1}, Difco) with appropriate sugars at 1% (w/v) and incubating anaerobically at 37 °C (Shklair & Keene, 1974).

Enzyme assays. *S. mutans* or *E. coli* cells were disrupted by sonication and the cell debris removed by centrifugation at 4 °C and 13600 *g* for 10 min; phage lysates were prepared as described by Russell *et al.* (1985). Protein concentration was determined with the Pierce protein reagent using bovine serum albumin as a standard. α -Galactosidase activity was determined either with whole cells or with cell-free extracts in 50 mM-Tris/HCl buffer, pH 8, containing appropriate substrate in the wells of a microtitre tray. With α -*p*-nitrophenyl galactoside (α -pNP-Gal, Sigma) the change in absorbance of the reaction mixture was followed at 405 nm using a Titertek plate reader. With 4-methylumbelliferyl α -D-galactoside as substrate, the fluorescence of released methylumbelliferone was measured at 460 nm (excitation at 380 nm) using a Perkin Elmer fluorimeter with microplate-reading attachment. When raffinose or melibiose was used as substrate, liberated galactose was quantified by coupling the reaction to galactose dehydrogenase at pH 8. The reaction mixture contained 50 mM-Tris/HCl (pH 8); raffinose or melibiose; NAD, 10 mM; galactose dehydrogenase (Sigma), 0.357 units ml^{–1}; cell-free extract protein, 35 μ g. Increase in absorbance at 340 nm with time was followed. The absorption coefficient of NADH was taken to be 6.22 $\times 10^3$ l mol^{–1} cm^{–1}.

Gel filtration. Samples were applied to a 30 cm $\times$ 1 cm column of Sepharose CL-6B-200 (Pharmacia) equilibrated with 50 mM-Tris/HCl (pH 7.5) containing 100 mM-NaCl. Eluted samples were collected into microtitre plates with a Gilson 203 fraction collector. The void volume was determined with Blue Dextran. Thyroglobulin (*M*_r 669000), apoferritin (443000), β -amylase (200000), alcohol dehydrogenase (150000), albumin (69000) and β -galactosidase (460000) from an extract of *E. coli* M2508 were used as *M*_r markers. β -Galactosidase was assayed as described for α -galactosidase but with *o*-nitrophenyl β -D-galactopyranoside (Sigma) as substrate. Protein concentration was determined as above.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was as described by Laemmli (1970).

Isoelectric focusing. This was done using ultrathin-layer polyacrylamide gels (Servalyt Precotes 3-10, Serva Feinbiochemica). Calibration was done with the Broad pI kit of coloured standard proteins supplied by BDH. α -Galactosidase activity was visualized by staining with 5-bromo-4-chloro-3-indolyl α -D-galactopyranoside (X- α -Gal) in 50 mM-sodium phosphate buffer, pH 6.5 (Tubb & Liljestrom, 1986).

Results

Cloning of *aga*

Screening of the recombinant gene library of the *S. mutans* chromosome in phage λ on sucrose-containing

Fig. 1. Partial restriction map of the cloned segment of *S. mutans* Ingbritt chromosome showing the position and orientation of *aga*, *gtfA* and *dexB*. The broad bands indicate the *EcoRI* fragment cloned in plasmid pSF106 and from which the nucleotide sequence data shown in Fig. 3 were obtained, the *AvalI* fragment subcloned to plasmid pSF110 and the *HindIII* fragment used for the gene-inactivation procedure.

medium resulted in the detection of plaques in which sucrase activity was manifested by the stimulation of growth of *E. coli* surrounding the plaques (Russell *et al.*, 1985). The activity in these plaques was found to be due to the expression of the genes *gtfA* or *scrB*, which were subsequently shown to encode sucrose phosphorylase (Russell *et al.*, 1988) or sucrose-6-phosphate hydrolase (Hayakawa *et al.*, 1986; Lunsford & Macrina, 1986) respectively. Some plaques also accumulated polymer and were found to express the *ftf* gene for fructosyltransferase (Aduse-Opoku *et al.*, 1989) or a *gtf* gene for glucosyltransferase as previously described for another species of oral *Streptococcus* (Gilpin *et al.*, 1985). To aid in the classification of these various types of sucrose-degrading recombinants, the plaques were rescreened on medium containing raffinose as carbon source. The α -linked trisaccharide raffinose is galactosyl-glucosyl-fructose and thus serves as a substrate for α -galactosidase, invertase and fructosyltransferase (the latter two enzymes can utilize either sucrose or raffinose). Of the recombinants expressing *gtfA* and so capable of degrading sucrose, approximately 50% could also attack raffinose (Ferretti *et al.*, 1988). However, none formed polymer, indicating that no fructosyltransferase activity was present; there was no additional action on sucrose beyond that due to the presence of *gtfA*, indicating that invertase was also not present. Lysates were therefore prepared from *E. coli* infected with each of a number of recombinant phages and these were all found to have α -galactosidase activity detectable with colorimetric or fluorimetric substrates. In order to investigate the properties of the enzyme expressed in *E. coli* from the cloned α -galactosidase (*aga*) gene, it was necessary first to determine the properties of α -galactosidase in *S. mutans*.

Properties of *S. mutans* α -galactosidase

By use of the sensitive fluorogenic substrate methylumbelliferyl α -D-galactopyranoside, it was possible to detect α -galactosidase activity in whole cells or cell-free extracts

Table 1. Induction of *S. mutans* α -galactosidase

S. mutans strain Ingbritt was grown to mid-exponential phase in a chemically defined medium, harvested and washed in 50 mM-Tris/HCl (pH 8.0). Each reaction mixture (2 ml) contained a cell suspension which had been adjusted to an OD₆₅₀ of 1.0, and 10 mM- α -pNP-Gal. Samples (300 μ l) were removed at timed intervals, centrifuged, and the absorbance read at 405 nm. Glucose-grown cells liberated 0.22 nmol *p*-nitrophenol per OD₆₅₀ unit per min. Values are means of triplicate determinations.

Growth substrate	Relative activity
Raffinose	35.2
Melibiose	18.5
Glucose	1.0
Fructose	0.8
Sucrose	0.4
Galactose	1.7
Maltose	1.4

of *S. mutans* grown on rich medium or on semi-defined medium in which no galactose-containing carbohydrates were present. Marked increases in activity were, however, observed when melibiose (galactosyl-glucose) or raffinose was present as carbon source (Table 1). These results on the induction of α -galactosidase in *S. mutans* are thus in agreement with the report of Barletta & Curtiss (1989). Despite the fact that α -galactosidase was induced in melibiose-containing medium, growth on this substrate as sole carbon source was only about half of that on glucose, galactose or raffinose (mean generation times of 4–4.5 h, as compared to 1.75–2 h). Since maximum activity was found in cell-free extracts of *S. mutans* grown in defined medium with raffinose as carbon source, these extracts were used as source of enzyme to determine the pH optimum and kinetic properties of α -galactosidase (see Table 2). Dialysed extracts retained full activity which was not enhanced by the addition of 10 mM-Mn²⁺, Mg²⁺, Cu²⁺, Fe²⁺ or Zn²⁺ or by 10 mM-EDTA. Activity was completely inhibited by exposure of extracts to 100 μ M-*p*-chloromercuriben-

zoic acid, suggesting that a thiol group was essential for activity.

Subcloning of the *aga* gene

A partial restriction map of the region of *S. mutans* DNA insert in recombinant phage λ IS42 is shown in Fig. 1. Preliminary results indicated that *aga* was located on the 4.48 kb *Eco*RI fragment and nucleotide sequencing of this fragment (see below) revealed the existence of a long open reading frame (2160 bp). This open reading frame is bounded by two *Ava*II sites, which allowed the construction of recombinant plasmids pSF110 and pSF111, in which the 2.7 kb *Ava*II fragment is inserted in opposite orientations downstream of the *lac* promoter region of pUC plasmid vectors. α -Galactosidase activity could be detected only in extracts of *E. coli* strain JM109 carrying pSF110, indicating that transcription of *aga* proceeded in the direction left to right shown in Fig. 1 and that expression required the presence of an *E. coli* promoter. No activity was detected in extracts of *E. coli* carrying pSF111, regardless of whether the culture had been exposed to IPTG or not.

SDS-PAGE of proteins from *E. coli* JM109 carrying pSF110 and exposed to 1 mM-IPTG for 3 h showed the induction of a polypeptide of M_r 80000 (Fig. 2). The isoelectric point of α -galactosidase in extracts of both *S. mutans* Ingbritt and *E. coli* JM109 carrying pSF110 was at pH 6.1.

In order to characterize the cloned α -galactosidase further, and remove the possibility of background activity from the host cell, pSF110 was introduced into *E. coli* strain M2508, which has a mutation in the *melA* gene coding for α -galactosidase (Schmitt, 1968). The α -galactosidase in extracts from the recombinant had properties indistinguishable from those found for α -galactosidase from *S. mutans* with regard to pH optimum, pI, and K_m for melibiose, raffinose and the synthetic substrates.

Properties of the cloned α -galactosidase

E. coli M2508 containing plasmid pSF110 was grown overnight in 200 ml LB medium with 1 mM-IPTG, harvested, washed with 50 mM-Tris/HCl buffer, pH 7.5, and resuspended in 10 ml of the same buffer. Cells were sonicated and the supernatant clarified by centrifugation. Cell-free extracts were used in all assays. K_m values were calculated from double reciprocal plots of substrate concentration against rate of reaction and are given in Table 2.

Fig. 2. SDS-PAGE of M_r standards (lane A), and extracts of *E. coli* JM109 (lane B) and *E. coli* JM109 carrying plasmid pSF110 (lane C), in which *aga* is orientated so as to be under control of the *lac* operon. The cultures were grown in the presence of 1 mM-IPTG for 3 h.

Table 2. Comparison of the properties of α -galactosidases

	<i>E. coli</i> MelA* (chromosome)	<i>E. coli</i> RafA† (plasmid)	<i>B. stearothermo-</i> <i>philus</i> ‡	<i>S. mutans</i>
Subunit M_r	50602	81188	~80000	82022
No. of subunits	2	4	ND	4
Cofactors	NAD, Mn^{2+}	none	none	none
pH optimum	8.1	7.2	6.5	6.5
K_m α -pNP-Gal	3 mM	0.14 mM	ND	4.4 mM
K_m melibiose	10 mM	3.2 mM	ND	9.14 mM
K_m raffinose	ND	60 mM	ND	196.8 mM

ND, Not determined.

* Burstein & Kepes (1971); Liljestrom & Liljestrom (1987); Nagao *et al.* (1988).

† Schmid & Schmitt (1976); Aslanidis *et al.* (1989).

‡ Ganter *et al.* (1988).

M G I V I K D N L F Y I H T K D S S L I	20	M V G L V I V L M T I A H L V I G L S M	380
ATGGCATTTGTTATAAAGATAACCTTTTATATACACACCAAGACTCATCAITGATT	60	ATGGTTGGTTGGTCATCGTTTGTGATGACAATAGCTCCTGGTGGTGGTTGGTCAATG	1140
I E E R D G D L L K H L G K K I E A Y	40	K K N L V V P L I A L S R K F M I E D Y	400
ATTGAAGAAGAGATGGTGGTCTTTTAAACATCTAGGTAAATAATGAAGCCTAT	120	AAGAAAACCTGGTGGTTCCTTGTAGCTTTATCAAGGAATTCATGATAGAGATTAC	1200
H F S N T V F E K D H A F S A N P V A D	60	S L A F G L N Q K W Y Q L T A N F I E L	420
CATTTTCCAACTACTGTTTGAAGAAGGATCATGCTTTTTCAGCTAATCCAGTGGGAC	180	AGTTTGGCCTTGGTTTGAACCAAGAAATGGTATCAGTTGACAGCAACCTTATAGAGCTC	1260
N R N Y S Y D T Q R Q I F G V H G F G D	80	I L I G S F R Q I S E S I L I A I Q L	440
AATAGAAATATAGTTATGATACACAAGACAAATATTTGGTGTTCATGGTTTGGTGAT	240	ATCCTGATTTGGTTCATTCAGGCAGATCAGCGAGAGACATCTTATCTCGCAATTCATTTG	1320
F R V P S L I L Q H D N N D L T R F K F	100	V L N L A N L D V V A Y I K T V L D K L	460
TTTAGAGTCCCTCACTAATCCCTTCAACATGATAATAATGATTTTAACACAGGTTTAAATTT	300	GTCCTGAATCTAGCTAATCTCGATGTAGTTGCTTATATAAAACTTCTTGTGATAAGCTG	1380
K K A K I I K G I K A R G L P N P H S	120	L T E N T I D Y V K W D Y N R N I T N I	480
AAGAAGGCTAAATATTAAGGGGGGATCAAGGCAAGAGGTTTGCTTAATCCACATAGC	360	CTGACAGAAATACGATTGATTTGTTAAATGGGACTACATCGCAATATAACCAATATT	1440
S E S A Q S L A L I L E D D L A K S R I	140	G N G R T Y L E T Q M Q S H A Y I L G L	500
AGTGAGAGTGCTCAAGCTTGAATCTTCTTACAGATGATTTGGCAAAATCGCGCAT	420	GGCAATGGCAGAACTTACTTGGAAACGCAATGCAGTCGATGCTTATATACTGGGTTG	1500
V S D Y T A Y D D N A T I S T F V K F E	160	Y D M F S Y L T T K H D K V L F E S C S	520
GTCTGTGATTACGGCTTATGATGATAATGCCACCATTTCAACCTTTCTCAAAATTTGAA	480	TATGATATGTTTCTTACTTAACAACAAACACGATAGTCTTGTGTTGATCCCTGTTCA	1560
N L S D K K P V I L H R A L S T M F D L P	180	G G G R N D L G M M C Y F P Q V W S S	540
AATTTATCTGATAAGCTGTATTCTTCATCGTCTTATCAACAATGTTTGTATTACCT	540	GGAGTGGCGGTGGCAACCTTTGGGATGATGTGTATTCTTCTCAAGTGTGGTCAAGT	1620
A S H Y D V I T F Q G A Y A R E K S L R	200	D N T D A I A R L P I Q Y G S S Y L Y P	560
GCCAGCATATGATGATAACTTTTCAAGGAGCTTATGTCGCGAGAGAGCCCTTCGG	600	GATAATACAGATGCTATTGCAAGACTGCCTATACAGTATGGTTCCTCTTATCTTTACCCA	1680
R H Q I E Q G L F K I G S N R G A S G H	220	T I S M G A H V S A V P N H Q M N R H T	580
CGCCACCATTTGAACGGTCTGTTAAATAGGCTCTAATCGAGGAGCTTCAGGACAT	660	ACTATTTCTATGGAGCCCTGTTTACGGGTTCCTAACCATCAATGAACCGTCAATACC	1740
A Q T P S L I L T E H D S N E F Y G E A	240	P L T T R A H V A M M G N L G Y E L D L	600
GCTCAAAACCCCTTCTTAATTAACAGAGCATGATAGCAATGAGTTTATGGGAGGCT	720	CCTCTTAAACACAGCTCATGTTGCTATGATGGGAAATTTAGGTTATGAATGGACTTA	1800
L A L Q L I Y S G N F Q A F V Q K N Q L	260	A V L T K N E K K A V A N Q I K H Y K K	620
TTGGCTTTACAATTAATCTATAGCGGAAATTTTCAAGCTTTTGTTCAAAAAATCAATTA	780	GCAGCTTTAAACCAAAATGAAAAAGGCTGTTGCCAATCAGATTAAACATTATAAAAG	1860
N V V R L G I G I N D D N F S W E L Q A	280	I R S V V Q F G K L Y R L I N P E V G I	640
AACGTGCTTCGCTTAGGCATCGGAATAAATGATGATAATTTTTCATGGGAACTCAGGCT	840	ATAAGTCTGTTGTACAATTTGGCAAGCTTTACCGTCTAATTAATCCGAAAGTTGGAAT	1920
N Q S F E T P V A L I T Y T D K G L T D	300	N E V A V Q Y T Y D N Q V L V T Y V R I	660
AATCAGAGTTTGGACACCTTAGCTTTTGATTAACCTACACTGATGAAGGATTAACGGAT	900	AATGAGGTAGCAGTGCATATACCTATGCAATCAGGTACTGGTACTATGTCCTGATTT	1980
L T Q E S H N F I K R H I I P K N F A N	320	Q S T I E M M E T T V K L R G L D A	680
CTGACACAAGAAGTCATAATTTATTAACGTCATATTTATTCCTAAGAAATTTTGCTAAT	960	CAATCAACCATAGATGATGGAGCAACTGTAAAGTTGAGAGGACTAGATGAGATGCC	2040
K E R P I L I N N W R Q H I L I L I G V	340	I Y V L I E T G Q A F S G A E L M Y A G	700
AAGGAAGTCTCTATCAATAATTTGGAGGCAACATATTTTGATTTTAAATCGGAGTC	1020	ATTTATGTTCTTTATAGAACTGGACAAGCTTTTTCAGGTGCGGAGTTAATGATGCGGA	2100
N C W G W Q M K P E N L A L N S L C L T	360	L T I D M P Q G D Y L S K Q Y Y F I K K	720
AATGTGGGGCTGGCAGATGAAGCCAGAAAACCTGGCATTTGACACTTTTGTGCTTGACG	1080	TTGACCATGATATGCGCAAGGTGATTATTGAGTAAACAATCTATTTTATATAAAG	2160

*
TAG

Fig. 3. Nucleotide sequence of the *aga* gene of *S. mutans* Ingbritt and the deduced amino acid sequence of its product. Amino acid residues and nucleotides are numbered on the right of the sequence.

The pH optimum was determined using the fluorogenic substrate in a series of citrate, Sorenson phosphate and Tris/HCl buffers. The enzyme was active in the range 5–7.5, with a pronounced maximum close to pH 6.5. The α -galactosidase exhibited higher activity in phosphate than in Tris/HCl buffer, activity at pH 7.5 being 1.8 times greater in phosphate than Tris. In contrast to the *E. coli* α -galactosidase (Burstin & Kepes, 1971), there were no discernible differences in α -galactosidase activity between extensively dialysed and non-dialysed cell-free enzyme preparations. Furthermore, addition of 10 mM-EDTA had no effect. Metal ions (Mg^{2+} , Mn^{2+} , Cu^{2+} , Fe^{2+} , Zn^{2+}), added to 10 mM, had no effect.

When cell-free extracts of *S. mutans* or *E. coli* carrying plasmid pSF110 were subjected to gel permeation chromatography, α -galactosidase activity eluted as a single symmetrical peak, with an apparent M_r close to 300 000. However, since electrophoresis in the presence of SDS indicated that the product of the *aga* gene has an M_r of 80 000, these results suggest that the active enzyme consists of a tetramer of four subunits which are dissociated by SDS.

Nucleotide sequence of *aga*

The nucleotide sequence of the *aga* gene is shown in Fig. 3. Analysis of the *aga* open reading frame reveals that it encodes a 720 amino acid protein with a predominantly hydrophilic character and a predicted M_r of 82 022. There is no evidence for the existence of a signal peptide resembling those found in proteins which are known to be secreted (von Heijne & Abrahmsen, 1989). Furthermore, the M_r of the *aga* gene product detected by SDS-PAGE in recombinant *E. coli* was 80 000 (Fig. 2), close to that predicted from the sequence data, and there was no difference in isoelectric points of α -galactosidase produced in *S. mutans* and in recombinant *E. coli*. It therefore appears that the *aga* gene product does not undergo post-translational modification and it can be concluded to have an intracellular location.

Inactivation of the *aga* gene in *S. mutans*

In order to inactivate the *aga* gene, we used a strategy similar to that employed by Perry *et al.* (1985) and Barletta *et al.* (1988) to inactivate *gtfA*. A 0.45 kb *Hind*III internal fragment from within the *aga* coding region was ligated to plasmid pVA891 also cut with *Hind*III. The recombinant plasmid was then introduced into *S. mutans* strain LT11 by transformation and erythromycin-resistant colonies were selected. Since pVA891 cannot replicate in *S. mutans*, resistant colonies are those in which the entire plasmid has inserted into the chromo-

Fig. 4. DIAGON plot comparing the primary amino acid sequences of the α -galactosidase encoded by the *S. mutans* *aga* gene and the α -galactosidase encoded by the *E. coli* *rafA* gene. The numbers of individual amino acid residues are shown on the axes.

some by homologous recombination between the fragment of *aga* in the plasmid and the chromosomal gene.

In order to confirm the insertion of the plasmid into the chromosome of mutant 516, chromosomal DNA from *S. mutans* strains Ingbritt, LT11 and 516 were digested with *Eco*RI, subjected to agarose gel electrophoresis and probed by Southern blotting with a fragment including *aga*. The ethidium-bromide-stained agarose gel showed no apparent differences in DNA banding patterns between *S. mutans* strains Ingbritt, LT11 and 516. However, chromosomal integration of pSF122 was confirmed following hybridization; the 4.48 kb *Eco*RI fragment detected by the probe in strain LT11 (identical to that in Ingbritt) was increased in size by about 0.7 kb in mutant 516. This increase was that predicted from the restriction map of pSF122.

Mutant strain 516 in which the *aga* gene had been inactivated was tested for the presence of α -galactosidase and for the ability to ferment glucose, fructose, galactose, raffinose, melibiose, sucrose, lactose, isomaltose or isomaltotriose. No α -galactosidase activity could be detected in the mutant, showing that *aga* had indeed been inactivated. Mutant 516 was unable to produce acid from melibiose, showed reduced acid production from raffinose and failed to ferment isomaltose or isomaltotriose. The fermentation of other sugars was unaffected.

Comparison with other α -galactosidases

The properties of *S. mutans* α -galactosidase are summarized in Table 2, along with those of α -galactosidases from *Bacillus stearothermophilus* (Ganter *et al.*, 1988), the enzyme encoded by the *E. coli* K12 *melA* gene (Burstin

& Kepes, 1971; Liljestrom & Liljestrom, 1987; Nagao *et al.*, 1988; Schmitt & Rotman, 1966) and that encoded by the Raf plasmids found in many wild isolates of *E. coli* (Schmid & Schmitt, 1976; Schmitt *et al.*, 1979). The *S. mutans* α -galactosidase shows few properties in common with the *E. coli* chromosomally-determined enzyme but does resemble the enzyme from *B. stearothermophilus* in size and lack of requirement for cofactors. Comparison of the deduced amino acid sequences of the various α -galactosidases revealed no regions of homology between the *S. mutans* and *E. coli* chromosomal enzymes but the central region of the *S. mutans* enzyme and that encoded by the Raf plasmid of *E. coli* did show strong homology (Fig. 4).

Discussion

Ability to ferment melibiose is characteristic of most isolates of *S. mutans* and this activity is dependent upon α -galactosidase. We show here that loss of α -galactosidase following insertional inactivation of the *aga* gene results in inability to ferment melibiose. Fermentation of raffinose is also reduced following loss of α -galactosidase, the residual activity presumably being due to the fructose moiety of raffinose being polymerized by fructosyltransferase to a fructan which is subsequently cleaved by fructanase to release free fructose. Inactivation of *aga* also leads to loss of ability to ferment the α -glucosides isomaltose and isomaltotriose. We have reported elsewhere (Russell *et al.*, 1991) that inactivation of the *dexB* gene also abolishes fermentation of isomaltose and isomaltotriose (but does not affect melibiose utilization). The results obtained with the inactivation of *aga* can be interpreted as a polar transcriptional effect on the downstream *dexB* gene and we have evidence that the entire region including *aga*, *gtfA* and *dexB* (Fig. 1) constitutes an operon-like arrangement which also includes a series of genes for a binding-protein-dependent transport system (Russell *et al.*, 1991).

α -Galactosidases are not widely known in bacteria, though they have been reported in *Streptococcus bovis* (Bailey, 1963) and *Streptococcus pneumoniae* (Li *et al.*, 1963) as well as in *E. coli* and *B. stearothermophilus*. They are, however, of considerable interest to plant biochemists and are attracting interest for their possible use in biotechnological processes. The properties of the *S. mutans* α -galactosidase resemble closely the α -galactosidases found in *B. stearothermophilus* and there is a strongly-conserved region of amino acid sequence in the centre of the molecules (R. Mattes, personal communication). This conserved region is also found within the α -galactosidase specified by the Raf plasmids of *E. coli* (Aslanidis *et al.*, 1989). The Raf plasmids confer the

ability to transport and metabolize raffinose, carrying genes for a permease, α -galactosidase and sucrose hydrolase (invertase). Aslanidis *et al.* (1989) suggested that the *raf* operon found on the plasmids had a modular construction, having picked up each of the genes from a different source. It is therefore interesting to note the strong similarity between the Raf-plasmid-determined and the *S. mutans* enzymes, though there appears to be no similarity between the products of the transport and sucrase genes from the two sources. It seems possible that the Raf plasmid α -galactosidase may have originated in a Gram-positive organism, since it shares properties with *S. mutans* and *B. stearothermophilus* enzymes and is quite unlike the chromosomally-determined enzyme in *E. coli* (Table 2).

The linkage of *aga*, *gtfA* and *dexB* raises the possibility that this region of the *S. mutans* chromosome may contain additional genes concerned with the metabolism or transport of carbohydrates. Evidence from gene-inactivation described here and by Barletta & Curtiss (1989) also shows that the control of the three enzyme-coding genes already identified is in some way coordinated; further nucleotide sequencing of this region should help elucidate the mechanisms operating.

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References

- ADUSE-OPOKU, J., GILPIN, M. L. & RUSSELL, R. R. B. (1990). Genetic and antigenic comparison of *Streptococcus mutans* fructosyltransferase and glucan-binding protein. *FEMS Microbiology Letters* **59**, 279–282.
- ASLANIDIS, C., SCHMID, K. & SCHMITT, R. (1989). Nucleotide sequences and operon structure of plasmid-borne genes mediating uptake and utilization of raffinose in *Escherichia coli*. *Journal of Bacteriology* **171**, 6753–6763.
- BAILEY, R. W. (1963). The intracellular α -galactosidase of a rumen strain of *Streptococcus bovis*. *Biochemical Journal* **86**, 509–514.
- BARLETTA, R. G. & CURTISS, R. (1989). Impairment of melibiose utilization in *Streptococcus mutans* serotype c *gtfA* mutants. *Infection and Immunity* **57**, 992–995.
- BARLETTA, R. G., MICHALEK, S. M. & CURTISS, R. (1988). Analysis of the virulence of *Streptococcus mutans* serotype c *gtfA* mutants in the rat model system. *Infection and Immunity* **56**, 322–330.
- BURSTEIN, C. & KEPES, A. (1971). The α -galactosidase from *Escherichia coli* K₁₂. *Biochimica et Biophysica Acta* **230**, 52–63.
- FERRETTI, J. J., GILMORE, K. S. & COURVALIN, P. (1986). Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2'-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. *Journal of Bacteriology* **167**, 631–638.
- FERRETTI, J. J., HUANG, T. T. & RUSSELL, R. R. B. (1988). Sequence analysis of the glucosyltransferase A (*gtfA*) gene from *Streptococcus mutans* Ingbritt. *Infection and Immunity* **56**, 1585–1588.
- GANTER, C., BOCK, A., BUCKEL, P. & MATTES, R. (1988). Production of thermostable, recombinant α -galactosidase suitable for raffinose elimination from sugar beet syrup. *Journal of Biotechnology* **8**, 301–310.

- GILPIN, M. L., RUSSELL, R. R. B. & MORRISSEY, P. (1985). Cloning and expression of two *Streptococcus mutans* glucosyltransferases in *Escherichia coli* K-12. *Infection and Immunity* **49**, 414–416.
- HAMADA, S. & SLADE, H. D. (1980). Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiological Reviews* **44**, 331–384.
- HAYAKAWA, M., AOKI, H. & KURAMITSU, H. K. (1986). Isolation and characterization of the sucrose 6-phosphate hydrolase gene from *Streptococcus mutans*. *Infection and Immunity* **53**, 582–586.
- VON HEIJNE, G. & ABRAHMSEN, L. (1989). Species-specific variation in signal peptide design. *FEBS Letters* **244**, 439–446.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LI, Y.-T., LI, S.-C. C. & SHETLAR, M. R. (1963). α -Galactosidase from *Diplococcus pneumoniae*. *Archives of Biochemistry and Biophysics* **103**, 436–442.
- LILJESTROM, P. L. & LILJESTROM, P. (1987). Nucleotide sequence of the *mefA* gene, coding for α -galactosidase in *Escherichia coli* K-12. *Nucleic Acids Research* **15**, 2213–2220.
- LOESCHE, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiological Reviews* **50**, 355–380.
- LUNSFORD, R. D. & MACRINA, F. L. (1986). Molecular cloning and characterization of *scrB*, the structural gene for the *Streptococcus mutans* phosphoenolpyruvate-dependent sucrose phosphotransferase system sucrose-6-phosphate hydrolase. *Journal of Bacteriology* **166**, 426–434.
- MACRINA, F. L., EVANS, P. R., TOBIAN, J. A., HARTLEY, D. L., CLEWELL, D. B. & JONES, K. R. (1983). Novel shuttle plasmid vehicles for *Escherichia coli*–*Streptococcus mutans*. *Gene* **25**, 145–150.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NAGAO, Y., NAKADA, T., IMOTO, M., SHIMAMOTO, T., SAKAI, S., TSUDA, M. & TSUCHIYA, T. (1988). Purification and analysis of the structure of α -galactosidase from *Escherichia coli*. *Biochemical and Biophysical Research Communications* **151**, 236–241.
- PERRY, D. & KURAMITSU, H. K. (1981). Genetic transformation of *Streptococcus mutans*. *Infection and Immunity* **41**, 722–727.
- PERRY, D., NILSEN, L. J. & KURAMITSU, H. K. (1985). Mapping of a cloned glucosyltransferase gene in *Streptococcus mutans*. *Infection and Immunity* **50**, 130–135.
- RUSSELL, R. R. B. & FERRETTI, J. J. (1990). Nucleotide sequence of the dextran glucosidase (*dexB*) gene of *Streptococcus mutans*. *Journal of General Microbiology* **136**, 803–810.
- RUSSELL, R. R. B., MORRISSEY, P. & DOUGAN, G. (1985). Cloning of sucrose genes from *Streptococcus mutans* in bacteriophage lambda. *FEMS Microbiology Letters* **30**, 37–41.
- RUSSELL, R. R. B., MUKASA, H., SHIMAMURA, A. & FERRETTI, J. J. (1988). *Streptococcus mutans* *gtfA* gene specifies sucrose phosphorylase. *Infection and Immunity* **56**, 2763–2765.
- RUSSELL, R. R. B., ADUSE-OPUKU, J., TAO, L. & FERRETTI, J. J. (1991). A binding protein-dependent transport system in *Streptococcus mutans*. In *Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci*. Edited by G. Dunne, P. Cleary & L. McKay. Washington, DC: American Society for Microbiology (in the Press).
- SCHMID, K. & SCHMITT, R. (1976). Raffinose metabolism in *Escherichia coli* K-12. Purification and properties of a new α -galactosidase specified by a transmissible plasmid. *European Journal of Biochemistry* **67**, 95–104.
- SCHMITT, R. (1968). Analysis of melibiose mutants deficient in α -galactosidase and thiomethylgalactoside permease II in *Escherichia coli* K-12. *Journal of Bacteriology* **96**, 462–471.
- SCHMITT, R. & ROTMAN, B. (1966). α -Galactosidase activity in cell-free extracts of *Escherichia coli*. *Biochemical and Biophysical Research Communications* **22**, 473–479.
- SCHMITT, R., MATTES, R., SCHMID, K. & ALTENBUCHER, J. (1979). Raf plasmids in strains of *Escherichia coli* and their possible role in enteropathogenicity. In *Plasmids of Medical, Environmental and Commercial Importance*, pp. 199–210. Edited by K. N. Timmis & A. Puhler. Amsterdam: Elsevier.
- SHKLAIR, I. L. & KEENE, H. J. (1974). A biochemical scheme for the separation of the five varieties of *Streptococcus mutans*. *Archives of Oral Biology* **9**, 1079–1081.
- TERLECKYJ, B., WILLETT, P. N. & SHOCKMAN, G. D. (1975). Growth of several cariogenic strains of oral streptococci in a chemically defined medium. *Infection and Immunity* **11**, 649–655.
- TUBB, R. S. & LILJESTROM, P. L. (1986). A colony-colour method which differentiates α -galactosidase-positive strains of yeast. *Journal of the Institute of Brewing* **92**, 588–590.
- WILBUR, W. J. & LIPMAN, D. J. (1982). Rapid similarity searches of nucleic acid and protein data banks. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 726–730.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13 mp18 and pUC19 vectors. *Gene* **33**, 103–119.