Biochemical and genetic analysis of Streptococcus mutans α-galactosidase

J. ADUSE-OPOKU,1 LIN TAO,2 J. J. FERRETTI2 and R. R. B. RUSSELL1*

1Hunterian Dental Research Unit, London Hospital Medical College, Turner Street, London E1 2AD, UK
2Department of Microbiology and Immunology, University of Oklahoma, Oklahoma City, Oklahoma 73190, USA

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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 glfA 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 μg 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 μg ml⁻¹), erythromycin (400 μg 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 laboratory equipment were purchased from Sigma or Boehringer Mannheim.

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

Abbreviation: α-PNP-Gal, α-p-nitrophenyl galactoside.
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 λL47.1, 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 λL47.1, a recombinant of bacteriophage λL47.1 previously shown to carry an insert of chromosomal DNA from *S. mutans* strain Ingbritt including the *aga* 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 Avel fragment. Such a fragment was therefore isolated from the original recombinant phage λL47.2 by preparative agarose electrophoresis, the ‘sticky ends’ filled by use of Klenow fragment of DNA polymerase and the fragment ligated into the Smal sites of plasmid vectors pUC18 and pUC19 to generate recombinant plasmids pSF110 and pSF111. Plasmid pSF106 was constructed by ligation of 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* LT1 to erythromycin resistance, essentially as described by Perry & Kuramitsu (1981). *S. mutans* LT1 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, LT1 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 μg) 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 × 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 (DuPont Chemicals). Filters were washed with 2 × SSC plus 0.2% (w/v) SDS for 2 × 15 min and then with 0.2 × SSC plus 0.2% (w/v) SDS for 2 × 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 Thioglucoside medium (24 g l−1, Difco) with appropriate sugars at 1% (w/v) and incubating anaerobically at 37 °C (Shklar & 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 13 600 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/HC1 buffer, pH 8, containing appropriate substrate in the wells of a microtitre tray. With α-nitrophenyl galactoside (α-nPGal, Sigma) the change in absorbance of the reaction mixture was followed at 405 nm using a Titretek plate reader. With 4-methylumbelliferone as substrate, the fluorescence of released 4-methylumbelliferyl α-D-galactoside 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/HC1 buffer, pH 8, raffinose or melibiose; NAD, 10 mM; galactose dehydrogenase (Sigma), 0.357 units ml−1; cell-free extract protein, 35 μg. Increase in absorbance at 340 nm with time was followed. The absorption coefficient of NADH was taken to be 6.22 × 103 mol−1 cm−1.

**Gel filtration.** Samples were applied to a 30 cm × 1 cm column of Sepharose CL-6B-200 (Pharmacia) equilibrated with 50 mM-Tris/HC1 (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, 669 000), apoferritin (443 000), β-amylase (200 000), alcohol dehydrogenase (150 000), albumin (69 000) and β-galactosidase (460 000) from an extract of *E. coli* M2508 were used as M, 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 β-D-galactopyranoside (N-x-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...
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 sucrase 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 *gtf* 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 (agar) 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 methylum-belliferyl α-D-galactopyranoside, it was possible to detect *α*-galactosidase activity in whole cells or cell-free extracts 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 μM-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 2542 is shown in Fig. 1. Preliminary results indicated that *aga* was located on the 4-48 kb EcoRI 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 *AvaII* sites, which allowed the construction of recombinant plasmids pSF110 and pSF111, in which the 2-7 kb *AvaII* 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*₅, 80006 (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, pl, and *Kₐ* 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. *Kₐ* 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, 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.](image)

<table>
<thead>
<tr>
<th>Subunit <em>M</em>&lt;sub&gt;c&lt;/sub&gt; (chromosome)</th>
<th><em>E. coli</em> MelA&lt;sup&gt;*&lt;/sup&gt;</th>
<th><em>E. coli</em> RafA&lt;sup&gt;†&lt;/sup&gt; (plasmid)</th>
<th><em>B. steaero-thermophilus</em>&lt;sup&gt;‡&lt;/sup&gt;</th>
<th><em>S. mutans</em>&lt;sup&gt;§&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>ND</td>
<td>50602</td>
<td>81 188</td>
<td>~80 000</td>
<td>82 022</td>
</tr>
<tr>
<td>No. of subunits</td>
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<td>4</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
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<td>none</td>
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</tr>
<tr>
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<td>6.5</td>
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<tr>
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<td>0.14 mM</td>
<td>ND</td>
<td>4.4 mM</td>
</tr>
<tr>
<td><em>Kₐ</em> raffinose</td>
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<td>3.2 mM</td>
<td>ND</td>
<td>9.14 mM</td>
</tr>
<tr>
<td><em>Kₐ</em></td>
<td>ND</td>
<td>60 mM</td>
<td>ND</td>
<td>196.8 mM</td>
</tr>
</tbody>
</table>

ND, Not determined.

* Burstein & Kepes (1977); Liljestrom & Liljestrom (1987); Nagao et al. (1988).
† Schmid & Schmitt (1976); Aslanidis et al. (1989).
‡ Ganter et al. (1988).
Fig. 3. Nucleotide sequence of the \textit{agA} gene of \textit{S. mutans} Ingbert 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 (Burstein & 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 (Mg2+, Mn2+, Cu2+, Fe2+, Zn2+), 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 Mr of 80000. However, since electrophoresis in the presence of SDS indicated that the product of the aga gene has an Mr of 82000, 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 Mr of 82022. 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 Mr of the aga gene product detected by SDS-PAGE in recombinant E. coli was 80000 (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 HindIII internal fragment from within the aga coding region was ligated to plasmid pVA891 also cut with HindIII. 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-

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

**Discussion**

Ability to ferment melibiose is characteristic of most isolates of \textit{S. mutans} and this activity is dependent upon \(\alpha\)-galactosidase. We show here that loss of \(\alpha\)-galactosidase following insertional inactivation of the \textit{aga} gene results in inability to ferment melibiose. Fermentation of raffinose is also reduced following loss of \(\alpha\)-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 \textit{aga} also leads to loss of ability to ferment the \(\alpha\)-glucosides isomaltose and isomaltotriose. We have reported elsewhere (Russell \textit{et al.}, 1991) that inactivation of the \textit{dexB} gene also abolishes fermentation of isomaltose and isomaltotriose (but does not affect melibiose utilization). The results obtained with the inactivation of \textit{aga} can be interpreted as a polar transcriptional effect on the downstream \textit{dexB} gene and we have evidence that the entire region including \textit{aga}, \textit{gtfA} and \textit{dexB} (Fig. 1) constitutes an operon-like arrangement which also includes a series of genes for a binding-protein-independent transport system (Russell \textit{et al.}, 1991).

\(\alpha\)-Galactosidases are not widely known in bacteria, though they have been reported in \textit{Streptococcus bovis} (Bailey, 1963) and \textit{Streptococcus pneumoniae} (Li \textit{et al.}, 1963) as well as in \textit{E. coli} and \textit{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 \textit{S. mutans} \(\alpha\)-galactosidase resemble closely the \(\alpha\)-galactosidases found in \textit{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 \(\alpha\)-galactosidase specified by the Raf plasmids of \textit{E. coli} (Aslanidis \textit{et al.}, 1989). The Raf plasmids confer the ability to transport and metabolize raffinose, carrying genes for a permease, \(\alpha\)-galactosidase and sucrose hydrolase (invertase). Aslanidis \textit{et al.} (1989) suggested that the \textit{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 \textit{S. mutans} enzymes, though there appears to be no similarity between the products of the transport and sucrose genes from the two sources. It seems possible that the Raf plasmid \(\alpha\)-galactosidase may have originated in a Gram-positive organism, since it shares properties with \textit{S. mutans} and \textit{B. stearothermophilus} enzymes and is quite unlike the chromosomally-determined enzyme in \textit{E. coli} (Table 2).

The linkage of \textit{aga}, \textit{gtfA} and \textit{dexB} raises the possibility that this region of the \textit{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**


