Intracellular α-Amylase of Streptococcus mutans

CHRISTINE L. SIMPSON and ROY R. B. RUSSELL*
Department of Oral Biology, Dental School, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom

Received 13 April 1998/Accepted 17 June 1998

Dental caries arises as a consequence of enamel demineralization by organic acid end products formed by metabolism of dietary carbohydrates by bacteria in dental plaque. The organism believed to be the principal etiological agent of dental caries is Streptococcus mutans, and high levels of S. mutans at a tooth site are indicative of a high risk of subsequent caries development (15). Factors thought to determine the levels of S. mutans include the ability of this species to ferment a wide range of substrates and to withstand conditions of low pH. S. mutans can also accumulate a glycogen-like intracellular polysaccharide (IPS) containing mainly α-1,4-linked glucose units (11). This stored IPS is believed to be of significance, in the absence of fermentable dietary carbohydrates, in production of acid which can lead to enamel demineralization and caries development (15). Factors thought to determine the levels of S. mutans include the ability of this species to ferment a wide range of substrates and to withstand conditions of low pH.

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While dietary availability of carbohydrates is thus the major influence upon levels of S. mutans, and since starch is a major constituent of the human diet, it is important to investigate its metabolism by S. mutans. There are conflicting bodies of evidence from epidemiological studies on the cariogenicity of starch (25), though there is increasing concern that modern high-temperature processes for manufacturing starch-based food products may increase their fermentability. In North African countries, where the diet is extensively starch based, high numbers of S. mutans are found in plaque, though the fact that caries levels are generally low in these countries suggests that the means by which starch encourages S. mutans accumulation is not related to its fermentability (40).

Dental plaque exhibits starch-degrading activity, though this ability may be largely due to bound salivary α-amylase (8). In one report, S. mutans was demonstrated to have some starch-degrading activity but the enzymes involved were not characterized (10). Another study indicated that utilization of starch by S. mutans was dependent on the addition of exogenous α-amylase (4). In this study, we report the nucleotide sequence of an intracellular α-amylase of S. mutans and our attempt to determine its function in the utilization of starch and in the synthesis and breakdown of IPS.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Wild-type S. mutans (strain LT11) (36) was grown without agitation in either Todd-Hewitt broth (Oxoid) with 0.5% yeast infusion (THYE), brain heart infusion (Oxoid), or the semidefined medium of Terlecky et al. (38) modified by the replacement of individual amino acids with 0.5% casein hydrolysate (26). Carbohydrates were added as required at 0.5% unless otherwise stated. When required, kanamycin (500 μg ml⁻¹) was used for selection in S. mutans, and ampicillin (100 μg ml⁻¹), erythromycin (400 μg ml⁻¹), or kanamycin (25 μg ml⁻¹) was used for selection in Escherichia coli JM83 (44). Plasmid pOB209, used in a previous study (30), contains regM and also contains the amy gene in its entirety. Plasmid pOB219, which contains amy alone, was produced by ligating a 2.3-kbp EcoRI-EcoRV fragment from pOB209 into pUC19 digested with SmaI (21). The resulting plasmid was transformed into S. mutans strain LT11 (36) and pOB219 was isolated from colonies containing the recombinant plasmid. Plasmid pOB219 could not be retransformed into the parent strain S. mutans LT11 (36) and the amylase activity in S. mutans LT11 was driven by the amy promoter (8). The amylase activity was induced by maltose but not by starch, and no acid was produced from starch. S. mutans can, however, transport limit dextrins and maltodextrins generated by salivary amylase, but inactivation of amy did not affect growth on these substrates or acid production. The amylase digested the glycogen-like intracellular polysaccharide (IPS) purified from S. mutans, but the amy mutant was able to digest and produce acid from IPS; thus, amylase does not appear to be essential for IPS breakdown. However, when grown on excess maltose, the amy mutant produced nearly threefold the amount of IPS produced by the parent strain. The role of Amy has not been established, but Amy appears to be important in the accumulation of IPS in S. mutans grown on maltose.

Sequencing upstream of the Streptococcus mutans gene for a CcpA gene homolog, regM, revealed an open reading frame, named amy, with homology to genes encoding α-amylases. The deduced amino acid sequence showed a strong similarity (60% amino acid identity) to the intracellular α-amylase of Streptococcus bovis and, in common with this enzyme, lacked a signal sequence. Amylase activity was found only in S. mutans cell extracts, with no activity detected in culture supernatants. Inactivation of amy by insertion of an antibiotic resistance marker confirmed that S. mutans has a single α-amylase activity. The amylase activity was induced by maltose but not by starch, and no acid was produced from starch. S. mutans can, however, transport limit dextrins and maltodextrins generated by salivary amylase, but inactivation of amy did not affect growth on these substrates or acid production. The amylase digested the glycogen-like intracellular polysaccharide (IPS) purified from S. mutans, but the amy mutant was able to digest and produce acid from IPS; thus, amylase does not appear to be essential for IPS breakdown. However, when grown on excess maltose, the amy mutant produced nearly threefold the amount of IPS produced by the parent strain. The role of Amy has not been established, but Amy appears to be important in the accumulation of IPS in S. mutans grown on maltose.

Conclusively, the study demonstrates the role of α-amylase in the metabolism of starch by S. mutans and its significance in the development of dental caries.
Acid production from IPS. *S. mutans* LT11 and the *amy* mutant were grown in THYE plus 2% glucose or maltose to stationary phase, and the cultures were then incubated at 37°C for a further 2 h. A 5-ml sample of culture was removed for determining IPS consumption, and the remaining cells were harvested, washed in 135 mM KCl, and resuspended in 135 mM KCl to an OD<sub>620</sub> of 1.0. Cells were harvested and resuspended in 1/10 volume of 135 mM KCl. The cell suspension was placed in a 37°C water bath, and the pH was adjusted to about 7.3 with 0.01 M NaOH. Incubation was continued at 37°C, and the pH was monitored with a model PHM290 pH-stat controller (Radiometer Copenhagen) over a 15-min period.

Purification of IPS. *S. mutans* LT11 and the *amy* mutant were grown in 200 ml of THYE or semidefined medium with 2% glucose or maltose for 24 h. IPS was extracted by incubation with 50 μl of α-amylase (1 mg/ml) for 1 h at 37°C. The cell suspension was boiled twice with cold distilled water. A freshly made solution of 30% (wt/vol) KOH was added to make a final volume of 10 ml, and the suspension was boiled for 90 min. Extracts were centrifuged (6,000 × g, 15 min), and the pellet was discarded. IPS was purified by two precipitations with an equal volume of 95% ethanol, with cooling to −80°C, and then lyophilized.

Nucleotide sequence accession number. The sequence shown in Fig. 1B has been assigned GenBank accession no. AF085987.

RESULTS

Sequence analysis of the *amy* gene. Plasmid pOB209 was used in a previous study where a homolog of CcpA in *S. mutans*, RegM, was identified (31). Nucleotide sequencing revealed an open reading frame downstream of *regM*, and a BLAST search (1, 9) revealed homology to amylase genes of *Streptococcus bovis* and *Bacillus* spp. The deduced protein sequence had a high degree of similarity to the amylase proteins of a number of other organisms, particularly to the intracellular amylase of *B. bovis*, with which it had 60% amino acid identity.

The gene, which we named *amy*, has the same polarity as *regM*, with 150 bp interposed between them (Fig. 1). A potential terminator of *regM* with a free energy value of −4.4 kcal was identified between the genes. Nucleotide sequencing confirmed that the plasmids contain the *amy* gene in its entirety. The nucleotide sequence of the *amy* gene was 1,461 bp in length and was preceded by a putative ribosome binding site (AGGAG) located 6 bp upstream of a methionine codon. A potential promoter was also identified, with a putative −10 (TATATT) sequence beginning at base 149 and a −35 (CAGT) sequence beginning at base 126 (Fig. 1B). A potential terminator with a free energy value of −3.8 kcal was also identified. Unlike genes encoding amylases in *Bacillus* spp., the *amy* gene is not preceded by a catalytically-active element (CRE) sequence as determined by using the consensus sequence of Hueck et al. (13). The deduced amino acid sequence defined a protein of 486 amino acids, with a calculated molecular weight of 56,347. A protein of this size with starch-hydrolyzing activity was detected in whole-cell extracts of *E. coli* carrying pOB209 after SDS-PAGE and incubation with starch. No signal peptide could be identified with the SignalP program of Nielsen et al. (19), and no starch-binding domain could be identified by using the consensus sequence defined by Svensson et al. (33). We were also unable to experimentally demonstrate any binding of the amylase to cornstarch, α-cyclodextrin, or β-cyclodextrin (data not shown).

Inactivation of *amy*. To determine the function of Amy in *S. mutans*, the *amy* gene was interrupted by the insertion of a kanamycin resistance cassette as described in Materials and Methods. The insertion was confirmed by Southern blot analysis, and the loss of starch-hydrolyzing activity was demonstrated by iodine staining after 2 days' growth on plates containing TH broth plus 1% starch agar (Fig. 2). Inactivation of *amy* was confirmed by amylase assays of cell extracts. Despite the apparent release of enzyme on starch-agar plates, amylase activity could not be detected in 65% ammonium sulfate precipitates of culture supernatants of either the wild type or the...
amy mutant. These results suggest that S. mutans has a single amylase activity which is intracellular and is encoded by the amy gene.

Regulation of amylase. The amylases of S. bovis have been reported to be induced by starch and maltose and catabolite repressed by glucose (3, 5, 28). In order to determine if this was the case in S. mutans, cell extracts were prepared from overnight cultures of S. mutans LT11 grown in THYE with the addition of starch or maltose. Starch had no effect on amylase activity, but the addition of maltose increased amylase activity two- to threefold (Fig. 3). To test for catabolite repression, glucose was included in the media. This did not result in a

FIG. 1. Sequence analysis of the amy locus. (A) Genetic organization of the amy region in S. mutans. Clones expressing amylase activity are shown, with the EcoRV site used for producing subclone pOB219 and the KpnI site used to inactivate the amy gene by insertion of a kanamycin resistance cassette. (B) Nucleotide sequence and deduced amino acid sequence of the amy region from the end of regM. Potential ribosome binding sites (rbs), -10 and -35 promoter elements, and terminators are indicated. The KpnI site used to inactivate the amy gene by insertion of a kanamycin resistance cassette is indicated.
reduction in amylase activity, indicating that the intracellular amylase is not catabolite repressed by glucose in *S. mutans*.

**Analysis of products.** TLC analysis was used to determine the range of substrates the cloned *amy* activity was able to hydrolyze. Cell extracts of *E. coli* carrying pOB219, containing the entire *amy* gene, hydrolyzed soluble starch, amylose, and amylpectin. The products of hydrolysis were sugars ranging from glucose to maltohexaose and larger maltooligosaccharides (Fig. 4). Cell extracts of *S. mutans* LT11 produced the same pattern of products from starch, whereas the *amy* mutant was unable to act on starch at all. Cell extracts of recombinant *E. coli* expressing *amy* also hydrolyzed IPS produced by *S. mutans*, giving the same range of products as starch hydrolysis (Fig. 4), but greater amounts of enzyme activity were required to achieve complete digestion. The pattern of products was not affected by whether IPS was formed in the presence of glucose or maltose or whether it was synthesized by *S. mutans* LT11 or the *amy* mutant.

**Effect of amy on growth and IPS production.** In order to determine whether loss of *amy* affected growth rates, the growth of the wild-type *S. mutans* strain, LT11, was compared to that of the *amy* mutant. Growth rates in semidefined media with glucose, maltose, or maltooligosaccharides as the sole carbon source were not affected by the *amy* mutation.

Growth of the *S. mutans* wild type in THYE with excess sugar (2% glucose or maltose) was also compared with that of the *amy* mutant. Although growth rates were unaffected by loss of *amy*, the *amy* mutant reached a higher OD$_{620}$ than the wild type when grown on maltose (Fig. 5). The IPS content of the cells was also monitored during exponential and stationary phases. The *S. mutans* wild type and *amy* mutant produced similar amounts of IPS when grown on excess glucose, but the *amy* mutant produced nearly three times as much IPS as the *S. mutans* wild type when grown in the presence of excess maltose.

**Acid production from extracellular carbohydrates.** The ability of *S. mutans* LT11 and the *amy* mutant to ferment carbohydrates was determined by monitoring the drop in pH of an unbuffered cell suspension in the presence of 0.5% exogenous carbohydrate. Both the wild type and the mutant produced acid in the presence of glucose, maltose, and maltooligosaccharides, but neither produced acid from starch (Fig. 6).

**Acid production from intracellular carbohydrate.** The ability of *S. mutans* LT11 and the *amy* mutant to ferment IPS was determined by monitoring the drop in pH of cells grown to stationary phase in excess glucose or maltose. The wild type and the *amy* mutant produced similar pH drops after prior growth in either 2% glucose or 2% maltose to accumulate IPS (Fig. 7). Determining the IPS content of cells from the same cultures during incubation at 37°C revealed that the production of acid was paralleled by the consumption of IPS (Fig. 7). The *amy* mutant retained the ability to digest IPS produced during growth on glucose or maltose. Despite much higher levels of IPS in the maltose-grown *amy* mutant culture, the rate of acid production and the final pH reached were unaffected (Fig. 7).

**DISCUSSION**

In this study a gene from *S. mutans* LT11 encoding an intracellular α-amylase has been sequenced and its product has been partially characterized. Genes encoding intracellular α-amylases have previously been reported for *E. coli* and *S. bovis*,...
and the α-amylase of *S. mutans* has 60% amino acid identity to the latter (24, 28, 29, 43). Although there has been some characterization of these activities, no clear physiological role for intracellular α-amylase has been established for either *E. coli* or *S. bovis*. However, in *S. bovis* inactivation of the gene encoding intracellular α-amylase resulted in growth rates of 15 to 20% of that of the wild type, leading to the postulation that the intracellular enzyme plays an important role in rapid cell growth (3). In contrast to these findings, inactivation of intracellular amylase in *S. mutans* did not affect growth, nor did it affect the rate of acid production from glucose, maltose, or maltooligosaccharides. Genes homologous to intracellular amylases can also be found in *Salmonella typhimurium* (24) and *Streptococcus pneumoniae* (14), and intracellular α-amylase activity has also been detected in *Streptococcus salivarius* (43), though for these organisms no information on function is available.

*S. mutans* has previously been reported to have extracellular amylase activity (10), on the basis of hydrolysis of starch in agar plates. The protein encoded by *amy* does not contain a signal peptide typical of secreted proteins and appears to be located predominantly intracellularly. However, it is responsible for the clearing of starch observed around colonies, because the mutant in which the *amy* gene had been insertionally inactivated was unable to hydrolyze starch in agar plates. The mechanism by which some of the amylase activity escapes the cell is unknown, and in liquid medium, *S. mutans* does not have sufficient extracellular amylase activity to enable it to produce acid from starch or to grow on starch as a sole carbon source.

In agreement with a previous finding that *S. mutans* required
exogenous α-amylase in order to hydrolyze starch (4), addition of salivary α-amylase (10 U/ml; Sigma) enabled *S. mutans* to produce acid from starch (data not shown). *S. mutans* can thus utilize limit dextrins from starch digestion, which can be taken up by the multiple sugar metabolism transport system (37).

The extracellular α-amylases from both *Bacillus* spp. and *S. bovis* are catabolite repressed by glucose (5, 12, 22). We at first thought that the *S. mutans* amylase was also catabolite repressed because no clearing was seen on starch-agar plates to which 1% glucose had been added (30, 31). However, determination of α-amylase activity in cell extracts of liquid cultures showed no evidence for catabolite repression. The effect seen on plates may therefore be due to some other factor, such as local pH or an effect on release of amylase from the cell. Catabolite-repressed genes in gram-positive bacteria are generally believed to have 14-bp palindromic sequences called CREs within or near their promoter regions, where binding of catabolite control proteins prevents transcription (13). CRE sequences could not be identified in the promoter region of the intracellular α-amylase gene of either *S. mutans* or *S. bovis* by the method of Hueck et al. (i.e., one mismatch to the consensus sequence within 200 bp of the translation start site [13]).

As *S. mutans* is unable to produce acid from starch, it seems unlikely that Amy plays a role in starch utilization. Furthermore, its spectrum of action on starch, amyllose, and amylopeptin is not substantially different from that of salivary amylase, so it would play no further role in the metabolism of low-molecular-weight starch degradation products once they are transported into the cell. The only high-molecular-weight substrate therefore available to amylase within the cell would be IPS, and it has previously been suggested that in *E. coli* the intracellular amylase AmyA may be involved in the breakdown of IPS (24). Purified AmyA from *E. coli* does show some activity against glycogen, although increased concentrations of enzyme and prolonged incubation times were required before digestion products were detected (24). A similar result was obtained with the cloned activity from *S. mutans*. The amylase is thus capable of degrading IPS, but this activity does not appear to be of physiological importance, because inactivation of *amy* had no effect on either the rate of breakdown or the rate of acid production from IPS.

The only clear difference between the *amy* mutant and the parent strain which we observed was in the amount of IPS accumulated during growth on maltose. This result seems paradoxical, since amylase can degrade IPS. When glucose is present in excess, IPS is synthesized from glucose-1-phosphate and ADP-glucose by the sequential action of ADP-glucose pyrophosphorylase and ADP-glucose-glycogen glucosyltransferase, a pathway which is found in a wide range of organisms, including *S. mutans* (2, 23, 32). Less is known about the synthesis of IPS from maltose. Some species, for example, *Streptococcus pyogenes*, can make IPS only from maltose (18), and the enzyme responsible is thought to be amylomaltase (22), which can synthesize a low-molecular-weight α-1,4-glucan chain from maltose. Another enzyme which might be involved is the transglucosylase identified in both *B. subtilis* and *Streptococcus mitis* by Walker (41, 42), which synthesizes higher-molecular-weight maltodextrins from maltotriose. Neither of these activities has yet been reported for *S. mutans*, but if they are present and contribute to the accumulation of IPS during growth on maltose, or during growth on starch when exogenous amylase is present, they provide a substrate for the intracellular amylase and hence provide an explanation for the greater accumulation of IPS observed in the *amy* mutant than in the wild-type *S. mutans*. The function of the amylase might thus be to trim or in some way modulate the accumulation of IPS. Further work will be required to characterize the structure of IPS in *S. mutans* grown on maltose in order to resolve this issue.
ACKNOWLEDGMENT

This work was supported by Medical Research Council grant G9505672PB.

REFERENCES

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