Expression of *Streptococcus mutans* Aspartate-Semialdehyde Dehydrogenase Gene Cloned into Plasmid pBR322

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Streptococcus mutans chromosomal DNA cloned into the vector plasmid pBR322 in *Escherichia coli* is able to complement the metabolic defect of an aspartate-semialdehyde dehydrogenase (EC 1.2.1.11) gene (*asd*) deletion in the host strain. We constructed two Asd⁺ recombinant plasmids, pYA570 and pYA571, containing 4.7 and 4.5 kilobases, respectively, of *S. mutans* chromosomal DNA inserted into the *Hind*III restriction endonuclease site of pBR322 in the same orientation. The *S. mutans* UAB62 Asd⁺ DNA did not hybridize with *E. coli* DNA which contained an intact *asd* gene, but did hybridize with *S. mutans* UAB62 chromosomal DNA. Derivative Asd⁺ plasmids were then constructed from pYA570. One, pYA574, had a 4.5 kilobase *S. mutans* insert DNA in the opposite direction from pYA570. In another, pYA575, the *S. mutans* insert DNA was reduced in size to 1.3 kilobases. It was seen that the orientation of the *S. mutans* DNA fragment inserted into the promoter region of the pBR322 tetracycline resistance (Tc^r) gene affected expression of Tc^r. Orientation of the *S. mutans* insert also affected the stability of the plasmid in certain *E. coli* strains. Restriction maps for pYA570, pYA571, pYA574 and pYA575 using the endonucleases *Eco*RI, *Bam*HI, *Hind*III, *Pst*I and *Sal*I were determined. Asd⁺ plasmid-directed protein synthesis was studied in *E. coli* minicells. The plasmids pYA570, pYA574 and pYA575 each produced large amounts of a protein, with a monomeric molecular weight of about 45000, that was distinct from both pBR322 and *E. coli* specified proteins: this protein is the *S. mutans asd* gene product. Smaller derivatives of recombinant plasmid pYA575 that were Asd⁻ allowed the location of the *S. mutans asd* gene promoter and the direction of transcription to be determined.

INTRODUCTION

The bacterial species *Streptococcus mutans* includes several genetically and serologically distinct groups (Coykendall, 1974, 1977) and plays a key etiological role in development of dental caries in animals and humans (Gibbons & vanHoute, 1975; Loesche *et al.*, 1975; Hamada & Slade, 1980). Genetic analysis of *S. mutans* pathogenicity is, however, limited since classical means of gene transfer such as transformation (Perry & Kuramitsu, 1981), transduction, and conjugation (LeBlanc *et al.*, 1978) are as yet poorly developed or non-existent. Different approaches have been undertaken to investigate the genetic and biochemical bases of pathogenicity of *S. mutans*, including construction of *S. mutans* plasmid cloning vehicles and transformation into *Streptococcus sanguis* (Challis) (Behnke & Ferretti, 1980; Macrina *et al.*, 1980). Work in our laboratory has included cloning of *S. mutans* genes into *Escherichia coli* K12 using the plasmids pBR322 (Bolivar *et al.*, 1977) and pACYC184

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(Chang & Cohen, 1978) and the cosmid pJC74 (Collins, 1979) as cloning vectors, followed by study of *S. mutans* gene organization and expression in the *E. coli* background (Hansen *et al.*, 1981; Curtiss *et al.*, 1982).

To study the expression of genetic information of the Gram-positive bacterium *S. mutans*, in the Gram-negative bacterium *E. coli*, we chose to clone the gene for an enzyme which is known to be intracellular in both bacteria. Aspartate- β -semialdehyde dehydrogenase (EC 1.2.1.11) is an enzyme common to the biosynthetic pathways of lysine, methionine and threonine (Umbarger, 1978). In this paper, we report on the cloning of the *S. mutans asd* gene into the *Hind*III restriction endonuclease site of pBR322, on the construction of Asd⁺ and Asd⁻ derivative recombinant plasmids, and on the expression of the *S. mutans asd* gene in *E. coli* K12 cells.

METHODS

Bacterial strains and growth media. Bacterial strains used are listed in Table 1. *Streptococcus mutans* was grown in the complex medium Brain Heart Infusion (Difco) broth at 37 °C without aeration. Liquid minimal media (ML; Curtiss, 1965) for growth of *E. coli* strains contained 0.5% (w/v) glucose and the following supplements per ml as needed: 50 μ g L-meso-diaminopimelic acid (DAP), 22 μ g L-histidine, 20 μ g L-isoleucine, 40 μ g DL-valine, 88 μ g L-lysine, 0.5 μ g biotin. Minimal agar (MA) contained 1.5% (w/v) agar. To grow *E. coli* χ 1849 and *E. coli* χ 2656 for minicell harvest, 0.5% (w/v) Casamino acids (Difco) replaced individual amino acids. Complex media for *E. coli* growth were L-broth (Lennox, 1955) and Penassay agar medium (PA, Difco), both supplemented with DAP when necessary. Antibiotic concentrations used for routine selection were (per ml): 25 μ g ampicillin (Ap, Lederle) and 25 μ g tetracycline (Tc, Lederle). To amplify plasmid DNA, cultures in the late-exponential phase were incubated for 16 h at 37 °C in the presence of chloramphenicol (Cm, Sigma) (170 μ g ml⁻¹).

Tetracycline resistance level. PA plates were inoculated with 100–300 cells (in exponential growth phase), incubated overnight and replica plated on to PA plates with 0, 1, 10, 25, 75, 100, 150 or 200 μ g tetracycline ml⁻¹.

Preparation of DNA. For plasmid isolation after amplification, one-litre bacterial cultures were lysed by the method of Guerry *et al.* (1973) modified by the addition of a freeze-thaw step after lysozyme digestion, to facilitate lysis. Plasmid DNA was purified from cleared lysates by two CsCl-ethidium bromide equilibrium density gradient centrifugations (Hansen *et al.*, 1981). After removal of ethidium bromide by isopropanol extraction, plasmid DNA was dialysed against 10 mM-Tris.HCl/1 mM-EDTA (pH 8.0) at 4 °C. When necessary, plasmid DNA was concentrated by precipitation with 2 vol. ethanol and 0.1 vol. 2 M-sodium acetate (pH 5.3) at -20 °C.

To obtain small amounts of DNA from 1 ml overnight cultures to screen for plasmid restriction endonuclease digestion patterns, two rapid cleared lysate techniques were used. One was the rapid alkaline extraction procedure of Birnboim & Doly (1979), and the second was a scaled-down protocol by J. P. Robeson (personal communication) of the Triton X-100 cleared lysis technique of Katz *et al.* (1973).

To prepare chromosomal DNA from *S. mutans*, cells from one-litre overnight cultures were pelleted, washed with 2 M-NaCl and suspended in a solution of 20% glucose in 25 mM-sodium phosphate buffer (pH 6.4). Bacterial cells were lysed by adding mutanolysin (kindly provided by Kanae Yokogawa, Daiinippon Pharmaceutical Co., Osaka, Japan) to a concentration of 1 mg ml⁻¹ and incubating for 10 min at 60 °C (Yokogawa *et al.*, 1974). To complete lysis, Sarkosyl (Sigma) was added to a final concentration of 0.5% (w/v). The whole cell lysate was mixed with CsCl to a density of 1.7 g ml⁻¹ and centrifuged to equilibrium in a Beckman Ti50 rotor at 37 000 rev. min⁻¹ for 48 h, at 15 °C. Collected chromosomal DNA was dialysed against 10 mM-Tris/10 mM-EDTA buffer (pH 8.0), treated with Proteinase K (Merck) (1 mg ml⁻¹) for 3 h at 37 °C and then recentrifuged on another CsCl gradient.

Enzymes. The restriction endonucleases *Eco*RI, *Sal*I, *Pst*I, *Hind*III and *Bam*HI were purchased from New England Biolabs. T4 polynucleotide ligase was the kind gift of C.K.K. Nair; we also used commercial T4 polynucleotide ligase (New England Biolabs) and *E. coli* DNA polymerase I (Boehringer-Mannheim). Conditions for digestion of DNA by restriction endonucleases were as described in the New England Biolabs catalogue. After digestion, DNA was heated for 10 min at 65 °C to inactivate restriction enzymes.

Formation of recombinant plasmid molecules. pBR322 plasmid DNA and *S. mutans* chromosomal DNA, digested separately with *Hind*III restriction endonuclease, were mixed at a ratio of 1 μ g pBR322 to 3 μ g *S. mutans* DNA. DNA ligase reactions were carried out in a 100 μ l mixture containing 50 mM-Tris.HCl (pH 7.6), 10 mM-MgCl₂, 20 mM-dithiothreitol and 1 mM-ATP: reaction mixtures containing 2.5 μ g plasmid and 7.5 μ g chromosomal DNA were incubated overnight at 12 °C with 0.3 units of T4 polynucleotide ligase; then the mixture was incubated at 65 °C for 5 min.

When recombinant plasmid DNA was restriction endonuclease digested and religated, we incubated 0.5–1 μ g DNA at 12 °C for 16 h in a 50 μ l reaction mixture with 0.1 units T4 polynucleotide ligase.

Table 1. *Bacterial strains*

Strain	Description*	Reference/source
<i>Escherichia coli</i> K12		
χ 289	λ^- <i>tte-1</i>	This laboratory
χ 1825	<i>supE42 tte-1</i> Δ 29[<i>bioH-asd</i>] λ^-	This laboratory
χ 1849	<i>tonA53 dapD8 minA1 purE41 supE42</i> Δ 40[<i>gal-uvrB</i>] λ^- <i>minB2 his-53 nalA25 metC65 tte-1</i> Δ 29[<i>bioH-asd</i>] <i>ilv-277</i> <i>cycB2 cycA1 oms-1 hsdR2</i>	This laboratory
χ 2656	χ 1849 that contains the pBR322 plasmid; Ap ^r Tc ^r	Bolivar <i>et al.</i> (1977)
V517	Contains multiple plasmids for size standards	Macrina <i>et al.</i> (1978)
<i>Streptococcus mutans</i>		
UAB62	Serotype c, Str ^r Rif ^r Mel ⁺ Raf ⁺	Derived from PS14 Str ^r

* The symbols for *E. coli* strains used are those described by Bachmann & Low (1980). Plasmid pBR322 confers resistance to ampicillin (Ap^r) and tetracycline (Tc^r). The *S. mutans* phenotypes are resistance to streptomycin (Str^r) and rifampicin (Rif^r), or ability to ferment melibiose (Mel⁺) and raffinose (Raf⁺).

Transformation. Ligated DNA, diluted to a concentration of 20–100 ng ml⁻¹ in 67 mM-CaCl₂/10 mM-Tris (pH 8.0) and put on ice for at least 10 min, was used to transform *E. coli* K12 strain χ 1849 by a method developed specifically for χ 1849 by M. Inoue, L. Alexander and R. Curtiss III (unpublished data). In this procedure, all sedimentation is gentle (7710 g for 10 min at 20 °C) and all resuspension is without vortexing. An 18 h, 37 °C culture of χ 1849 in 5 ml L-broth plus DAP was diluted 1:10 into 20 ml L-broth plus DAP and incubated for about 3 h with shaking at 37 °C, to an absorbance of 0.4 at 600 nm. Cells were sedimented and resuspended in 10 ml 25 mM-KCl/10 mM-Tris.HCl buffer (pH 8.0). After 5 min at 20 °C, cells were sedimented and resuspended into 10 ml 100 mM-CaCl₂/10 mM-Tris.HCl buffer (pH 8.0), and held at 20 °C for 20 min. Cells were then sedimented and resuspended in 10 ml 67 mM-CaCl₂/10 mM-Tris.HCl (pH 8.0): 0.5 ml of these cells were put into a clean 13 × 100 mm Pyrex tube on ice for at least 2 min. Using a chilled pipette, 200 μ l chilled cells were mixed with 100 μ l chilled DNA and incubated at 4 °C for 20 min. The transformation mixture was then heat shocked at 42 °C for 2 min, and held at 20 °C for 10 min. To allow phenotypic expression, 0.9 ml L-broth plus DAP was added, followed by incubation at 37 °C for 90 min. Samples (0.1 ml) were spread on to freshly made plates of PA plus DAP and antibiotic. The use of overly dry plates or the practice of spreading to dryness were both avoided since either reduced transformant yield 100- to 1000-fold.

Gel electrophoresis and DNA size determinations. DNA was electrophoresed in various types of slab gels: 1.2%, 1% or 0.8% (w/v) agarose (Sigma), agarose acrylamide (0.7% and 2%, w/v) or 5% (w/v) acrylamide. Running buffer was 40 mM-Tris, 5 mM-sodium acetate and 1 mM-EDTA adjusted to pH 7.6 with acetic acid (Hayward & Smith, 1972). After ethidium bromide staining, the gels were photographed using u.v. light, Polaroid T57 film and a red filter. Molecular weight standards for supercoiled plasmid DNA were pBR322 (Sutcliffe, 1978) and the plasmids from strain V517 (Table 1). Molecular weight standards for linear restriction endonuclease fragments of DNA were chosen to bracket the sizes of the unknown fragments: we used variously bacteriophage λ DNA digested with *EcoRI* or *HindIII* and double-stranded ϕ X174 DNA digested with *HaeIII*. Standard curves were drawn as plots of molecular weight versus relative mobility (supercoiled DNAs were plotted on double-logarithm and linear DNAs on semi-logarithm paper). Plasmid sizes were measured both as intact molecules and as the summation of linear fragments. For mapping of restriction endonuclease sites, various double digestions were performed.

Southern blot hybridization. Chromosomal or plasmid DNA electrophoresed through 0.8% agarose gels was transferred to nitrocellulose filters and hybridized with radioactive probe DNA as described by Southern (1975). Probe plasmid DNA was labelled with [α -³²P]dATP (New England Nuclear) by the nick translation method of Rigby *et al.* (1977). Hybrids were visualized by autoradiography.

Minicell protein analysis. Minicells of *E. coli* χ 1849 or plasmid-containing χ 1849 derivatives were isolated by standard methods (Frazer & Curtiss, 1975): minicells from 50 ml of a late-exponential phase culture were purified by one differential centrifugation and two velocity centrifugations in discontinuous sucrose gradients. Minicells were incubated for 20 min at 37 °C in 0.6 ml ML without DAP and then labelled by the addition of 2 μ Ci ¹⁴C-labelled amino acid mixture [New England Nuclear, 0.1 mCi ml⁻¹ (3.7 MBq ml⁻¹)] followed by incubation for 20 min. Labelled minicells were pelleted, washed once with cold buffered saline with gelatin (Curtiss, 1965), suspended in 50 μ l sample buffer [3% sodium dodecyl sulphate (SDS), 5% β -mercaptoethanol, 10% glycerol, 0.1% bromophenol blue, 62.5 mM-Tris.HCl, pH 6.8] and heated at 100 °C for 5 min. Proteins from disrupted minicells were fractionated on SDS–polyacrylamide gels (6% stacking gel, 10% separating gel) by the method of Laemmli & Favre (1973). After fixing, the gels were stained with 0.01% Coomassie Blue in 50% methanol and 9% acetic acid, destained with 7% acetic acid in 5% methanol, impregnated with a scintillator (Enhance, New England Nuclear), dried and fluorographed. Protein molecular weight standards employed were from BioRad.

RESULTS

Cloning of the S. mutans aspartate semialdehyde dehydrogenase gene into pBR322. *S. mutans* UAB62 genes were 'shotgun' cloned into the single *Hind*III endonuclease restriction site of the ampicillin and tetracycline antibiotic resistance (Ap^rTc^r) plasmid, pBR322. Insertion of DNA at this site often prevents the expression of Tc^r (Bolivar *et al.*, 1977). The frequency of transformation into *E. coli* χ 1849 cells with ligated DNA was 1×10^6 Ap^r transformants per μ g pBR322 DNA. Two of approximately 30000 Ap^r transformants, selected on PA agar with ampicillin, were also Asd⁺, as evidenced by their ability to grow on minimal agar with ampicillin but lacking threonine. These two Asd⁺ Ap^r clones were also Tc^s. The recombinant plasmids from the two Asd⁺ clones designated pYA570 and pYA571, were both larger than pBR322, and had DNA inserts at the *Hind*III site of sizes 4.7 and 4.5 kilobases (kb), respectively.

Genetic complementation of the asd mutation in E. coli χ 1825. In *E. coli* strain χ 1825, unlike χ 1849, only a single mutation, the $\Delta 29[*bioH*-*asd*]$ deletion, affects the diaminopimelic acid (DAP), methionine and threonine biosynthetic pathways. Growth of χ 1825 on minimal agar requires DAP, methionine and threonine. When χ 1825 was transformed by pYA570 plasmid DNA with selection for growth in the absence of threonine, methionine and DAP, either singly or in combinations, transformants were obtained at similar frequencies in each case. These frequencies were the same as the frequency of Ap^r transformants selected on PA plates with DAP.

A generation time of 1.5 h was obtained for both χ 1825 and χ 1825(pYA570) cells on minimal medium supplemented with glucose, biotin, DAP, methionine, and threonine, as well as for χ 1825(pYA570) cells on minimal medium supplemented only with glucose and biotin. These results suggested that *S. mutans* DNA cloned into the *Hind*III cleavage site of pBR322 provided a function which substituted for the deleted *asd* gene in *E. coli* χ 1825.

Hybridization of pYA570 insert DNA with S. mutans UAB62 DNA. The following Southern filter blot hybridization experiment revealed that the specific nucleotide sequence which encodes aspartate- β -semialdehyde dehydrogenase in *S. mutans* UAB62 was unrelated to the *E. coli asd* gene sequence and only encoded for similar function. *Hind*III-digested, ³²P-labelled probe pYA570 DNA hybridized with *Hind*III-digested pYA570 DNA and *S. mutans* UAB62 chromosomal DNA, but not with *E. coli* χ 1825 or χ 289 chromosomal DNAs (χ 289 has an intact *asd* gene). This indicated that the inserted fragment is a part of the *S. mutans* UAB62 genome (Fig. 1). In a control experiment, radioactive *Hind*III-digested pBR322 plasmid DNA did not hybridize with either *E. coli* or *S. mutans* chromosomal DNA (data not shown); thus the hybridization seen for pYA570 was all due to the inserted 4.7 kb *S. mutans* DNA.

Characterization of Asd⁺ recombinant plasmid DNA. The relative locations of *Pst*I, *Eco*RI, *Bam*HI and *Hind*III endonuclease cleavage sites have been mapped (see Methods). The *S. mutans* DNA in both recombinant plasmids is inserted into the *Hind*III restriction site in the same orientation relative to pBR322, and pYA570 differs from pYA571 by having an extra 0.2 kb *Hind*III fragment (Fig. 2). Neither insert DNA is cut by *Sal*I endonuclease.

To determine whether the *S. mutans* DNA responsible for the Asd⁺ phenotype is expressed in *E. coli* cells in both orientations in pBR322, pYA570 DNA was digested with *Hind*III, ligated and transformed into *E. coli* χ 1825 cells with selection for Ap^rAsd⁺ transformants. Half of fifty transformant colonies screened were found to be Tc^r. Plasmids from four Ap^rTc^rAsd⁺ clones and from four Ap^rTc^sAsd⁺ clones all had molecular sizes of about 9 kb, similar to the original pYA570 plasmid. Restriction endonuclease analysis with *Eco*RI or *Pst*I of purified plasmid DNA from one Ap^rTc^rAsd⁺ clone, designated as pYA574, revealed that the *S. mutans* DNA was inserted into pBR322 in the opposite orientation from pYA570 (Fig. 2). The fact that *S. mutans* DNA cloned in both orientations gave an Asd⁺ phenotype

Fig. 1. Hybridization of pYA570 plasmid DNA with *S. mutans* UAB62, *E. coli* χ 289 and *E. coli* χ 1825 chromosomal DNA. Unlabelled chromosomal and plasmid DNAs were digested with *Hind*III restriction endonuclease, electrophoresed through 0.8% agarose, denatured and transferred to nitrocellulose paper. Probe DNA was pYA570 digested with *Hind*III and labelled with [α - 32 P]dATP by nick translation. Hybridization was detected by autoradiography. (a) Photograph of *Hind*III-digested *S. mutans* UAB62 chromosomal DNA (lane 1) and plasmid pYA570 DNA (lane 2) in the agarose gel before transfer (there is only one apparent band since pBR322 and the insert are nearly the same size). (b) Autoradiograph of pYA570, *E. coli* and *S. mutans* DNA after hybridization with 32 P-labelled pYA570 DNA. Unlabelled DNA was as follows: lane 1, χ 289; lane 2, χ 1825; lane 3, UAB62, lane 4, pYA570.

suggested that a functional *S. mutans* promoter for the *S. mutans asd* gene was recognized by *E. coli* RNA polymerase. Furthermore, in one orientation (pYA574) the *S. mutans* DNA fragment provided a promoter function which substituted for the disrupted promoter of the pBR322 *Tc^r* gene(s). Unlike other *Asd⁺* plasmids we have constructed (above and below), pYA574 was unstable under non-selective growth conditions in χ 1849, but not in χ 1825 (data not shown). The reason for this strain-specific, orientation-dependent instability is unclear.

Construction of a low molecular weight Asd⁺ derivative of pYA570. To determine the minimum size of *S. mutans* DNA required for expression of the *Asd⁺* phenotype, we next attempted to reduce the size of *S. mutans* DNA cloned into pBR322. When we subcloned the 2.8 kb pYA570 *Bam*HI fragment into the *Bam*HI cleavage site of pBR322, all *Ap^rTc^s*

Fig. 2. Physical map of *Asd*⁺ and *Asd*⁻ recombinant plasmids showing cleavage sites for *Eco*RI (E), *Hind*III (H), *Pst*I (P) and *Bam*HI (B) restriction endonucleases. The construction and characterization of these plasmids was described in the text; sizes of various deletions are indicated by open boxes. Note that the size of the *Eco*RI-*Hind*III deletion in pBR322 DNA, only 29 base pairs, has been exaggerated on this map for visibility. Each plasmid is represented as a linear map, with the ends being a *Hind*III restriction site. Note that the pBR322 DNA is inserted in opposite orientation for the lower four plasmids as compared with the upper two. Distances in kilobases (kb) are shown for pYA570. All of the *Eco*RI, *Hind*III, *Bam*HI and *Pst*I sites present in the *S. mutans* insert are marked on pYA570; for clarity, only the restriction sites relevant to plasmid construction are indicated in the *S. mutans* insert DNA of the other five plasmids. pBR322 DNA is represented by a broken thick line and *S. mutans* DNA by a solid thick line. A thinner broken line with an arrow represents transcription regions for ampicillin and tetracycline resistances (*Ap*^r, *Tc*^r) in pBR322 and for aspartate- β -semialdehyde dehydrogenase (*asd*) in *S. mutans* DNA. The bottom *asd* transcription arrow represents a hypothetical fused protein (see Fig. 3).

transformants isolated had an *Asd*⁻ phenotype. Examination of 10 of these bacterial clones revealed that all contained a recombinant plasmid with an insert of about 2.8 kb, as was expected. It was concluded that one of the *Bam*HI cleavage sites was most probably located inside the *asd* structural gene or promoter. This conclusion is also compatible with our inability to construct *Asd*⁺ recombinant plasmids in a *Bam*HI 'shotgun' cloning of *S. mutans* chromosomal DNA.

When we subcloned portions of the *S. mutans* insert DNA, made by digesting pYA570 completely with *Hind*III and incompletely with *Eco*RI, into pBR322 plasmid DNA which was completely digested with *Hind*III and *Eco*RI, we obtained three transformant bacterial clones that were *Ap*^r*Tc*^r*Asd*⁺ and carried plasmid DNA of the same reduced size, in comparison with pYA571. Plasmid DNA from one of these clones, designated pYA575, was isolated, purified and mapped as described in Methods. The pYA575 recombinant plasmid had a molecular size of 5.7 kb, having lost the 2.7, 0.5 and 0.2 kb DNA fragments of pYA570 generated by *Hind*III and *Eco*RI digestion. Thus no more than about 1.3 kb of *S. mutans* DNA was required to code for the *Asd*⁺ function necessary to substitute for the deleted *asd* gene in *E. coli* χ 1825 (Fig. 2).

The expression of *Tc*^r by pYA575 might be due to transcription initiation at a promoter sequence on the *S. mutans* inserted DNA. However, the resistances to tetracycline of χ 2656, which contains pBR322, and χ 1849(pYA575), determined as a 50% inhibition of growth on

plates, were both the same: between 100 and 150 μg tetracycline ml^{-1} . The $\chi 1849$ plasmid-free control and $\chi 1849(\text{pYA570})$ both showed a tetracycline minimal inhibitory concentration of about 1 μg ml^{-1} .

Inactivation of the cloned asd gene. We next constructed two Asd^- derivatives of pYA575. One type was obtained by *Pst*I digestion of pYA575 followed by religation and selection for Tc^r transformants in $\chi 1849$. These Tc^r transformants were $\text{Ap}^s \text{Asd}^-$. Plasmid DNA from several clones examined all had a molecular size of about 4.7 kb, and only one *Pst*I endonuclease site. One representative plasmid was designated pYA576 (Fig. 2). Asd^- derivatives of pYA575 of the second type were constructed by *Hind*III and *Bam*HI digestion of pYA575 followed by religation. Plasmid DNA from five $\text{Ap}^r \text{Tc}^s \text{Asd}^-$ transformants examined had a molecular size of about 4.7 kb. One of these plasmids was designated pYA577. pYA577 DNA had no cleavage sites for the *Hind*III restriction enzyme and had only one *Bam*HI endonuclease site. Restriction maps of both deletion plasmids are given in Fig. 2.

Identification of protein encoded by Asd^+ recombinant plasmids. A comparison of protein synthesis in minicells that contained only the vector plasmid pBR322 with those that contained pYA570, pYA574 or pYA575 DNA allowed us to determine the specific polypeptide(s) associated with cloned *S. mutans* DNA: an autoradiograph of radioactively labelled minicell proteins after SDS–polyacrylamide gel electrophoresis is shown in Fig. 3. First, several protein bands were present in plasmid-free minicells: they were due to stable mRNA (Levy, 1975) (Fig. 3, lane 1). The cloning vector pBR322 specified the synthesis of several proteins (Fig. 3, lane 2). The most prominent bands corresponded to three β -lactamase proteins in the range 25 000–31 000 daltons. The β -lactamase gene promoter initiates transcription more efficiently than the Tc^r gene promoters (Stüber & Bujard, 1981), so that the pBR322-directed minicell-protein profile is dominated by the β -lactamase proteins (Sutcliffe, 1978). But the 34 000 dalton protein involved in Tc resistance, which disappears when pBR322 plasmid DNA has foreign DNA inserted in the *Bam*HI or *Sal*I restriction site (Tait & Boyer, 1978), was also visible (Fig. 3, lane 2). This protein migrates only slightly faster than one of the stable mRNA proteins.

The Asd^+ recombinant plasmids specified a novel polypeptide of monomeric molecular weight of about 45 000 (Fig. 3, lanes 5, 6, 7) which must be encoded by the 1.3 kb *S. mutans* DNA common to all (Fig. 2). This unique band was also visible after SDS–polyacrylamide gel electrophoresis and Coomassie blue staining of proteins isolated from unlabelled *E. coli* $\chi 1849$ cells carrying pYA570 (Curtiss *et al.*, 1982). Notably, Asd^+ recombinant plasmids gave rather faint bands for β -lactamase when compared with pBR322 itself (Fig. 3). This observation suggested that the *S. mutans asd* promoter is strong and may compete with the Ap^r and Tc^r gene promoters for the limiting amounts of RNA polymerase in minicells (Frazer & Curtiss, 1975). Stüber & Bujard (1981) also observed that cloned foreign DNA carrying an efficient promoter can compete with promoters of pBR322.

A comparison of polypeptide synthesis in minicells directed by the Asd^- plasmids pYA576 and pYA577 (Fig. 3) allowed us to determine the location of the *S. mutans asd* gene promoter and direction of *asd* gene transcription shown in Fig. 2. In both of these cases, deletion of *S. mutans* DNA correlated with disappearance of the 45 000 molecular weight polypeptide. pYA576 specified only moderate synthesis of the 34 000 dalton, pBR322-encoded tetracycline resistance protein (this protein appears just slightly below the lower stable mRNA protein: compare with plasmid-free control). β -Lactamase proteins were not made by this Ap^s plasmid (Fig. 3, lane 3). These results with pYA576 suggested that the *asd* promoter was located on the 0.20 kb *Eco*RI–*Pst*I fragment of pYA575. When we analysed protein synthesis directed by pYA577, we found that pYA577 produced a novel, probably fused, protein of smaller monomeric molecular weight (32 000) in addition to the β -lactamase proteins expected (Fig. 3, lane 4). We assume that this protein is a fused product since it is larger than coding capacity

Fig. 3. Identification of proteins encoded by *Asd*⁺ recombinant plasmids. Lanes 1 to 7 are fluorographs showing stable chromosomal mRNA and plasmid-specific proteins synthesized by purified minicells: 1, χ 1849; 2, χ 1849(pBR322); 3, χ 1849(pYA576); 4, χ 1849(pYA577); 5, χ 1849(pYA570); 6, χ 1849(pYA574); 7, χ 1849(pYA575). Molecular weights of labelled proteins (see Results) were estimated from comparison of their mobility with that of unlabelled proteins of known molecular weights (see Methods).

of the *S. mutans* insert in pYA577. The proposed location of the *S. mutans asd* gene promoter and direction of transcription are shown on Fig. 2. The coding capacity of the *S. mutans* DNA insert in pYA575 (about 430 amino acids), the molecular size of the *asd* gene product (about 400 amino acid moieties) and the fact that the pYA576 plasmid does not specify synthesis of truncated or fused protein, all strongly support the hypothesis that the *asd* gene promoter is located in the 0.2 kb *EcoRI*–*PstI* *S. mutans* DNA fragment adjacent to the *EcoRI* restriction site and that direction of transcription of *asd* is towards the *HindIII* recognition site.

DISCUSSION

We have shown that chromosomal genetic information (the *asd* gene) from *S. mutans*, a Gram-positive bacterium, can be stably maintained (depending on the host strain) in the pBR322 vector plasmid in either of the two possible orientations and can complement an *E. coli* K12 *asd* deletion mutation. Our smallest Asd⁺ chimeric plasmid contained only about 100 base pairs of DNA more than is necessary to encode the approximately 45 000 molecular weight polypeptide which we believe to be the *asd* protein. Aspartate- β -semialdehyde dehydrogenase has been characterized after purification from *E. coli* cells and commercial baker's yeast: the molecular weight of the *E. coli* enzyme is approximately 70 000 (Hegeman *et al.*, 1970), while yeast aspartate- β -semialdehyde dehydrogenase has a molecular weight of 156 000 and consists of four identical subunits of molecular weight $41\,000 \pm 4000$ (Holland & Westhead, 1973). We do not know whether the *S. mutans* aspartate- β -semialdehyde dehydrogenase holoenzyme is multimeric.

In protein synthesis experiments the *asd* protein surprisingly appeared in great excess compared with products of the pBR322 ampicillin or tetracycline resistance genes. No other *S. mutans* gene cloned in *E. coli* in this laboratory has had as high a level of expression as the *asd* gene. Examination of the *S. mutans asd* promoter by RNA polymerase binding assay and DNA sequencing is under way in this laboratory to determine the cause of its strong activity. The copy number of the Asd⁺ plasmids was not different from pBR322 (data not shown).

The *asd* promoter sequence appears to be located on the 0.20 kb *EcoRI*–*PstI* fragment (Fig. 2). Evidence for this came from two derivative plasmids, both Asd[–], which have part of the original *HindIII asd* gene fragment deleted. One, lacking the 0.6 kb *BamHI*–*HindIII* fragment downstream of the promoter, made a novel fused protein of only 32 000 daltons: presumably aspartate- β -semialdehyde dehydrogenase which lacks part of the C-terminal end. The other, lacking the 0.2 kb *EcoRI*–*PstI* fragment, produced no detectable *S. mutans* protein. Preliminary sequencing data also support this promoter location (G. Cardineau, unpublished).

Sutcliffe (1978) reported that cloning foreign DNA into the pBR322 *HindIII* restriction site does not always change the plasmid's phenotype to Tc^s. The *S. mutans* UAB62 *asd* gene inserted in one of the two possible orientations (pYA574, pYA575) also maintained the Tc^r phenotype. pBR322 DNA has an excellent promoter sequence near the *HindIII* recognition site (Sutcliffe, 1978): the AT-rich RNA polymerase binding site (Pribnow-box) is located downstream from the *HindIII* cleavage site, but the 'minus 35' region necessary as the RNA polymerase recognition site is located upstream from the *HindIII* cleavage site. Expression of Tc^r by one orientation of the *asd* insert DNA may be due to substitution for only the 'minus 35' region, or alternatively, cloned *S. mutans* DNA may provide a complete promoter upstream of the *HindIII* site. In the latter alternative, there would have to be a second promoter in addition to the *asd* promoter, since deletion of the *asd* promoter (pYA576) did not affect tetracycline resistance. Tetracycline minimal inhibitory concentration data for pBR322 and pYA575 plasmid-carrying χ 1849 cells did not help us choose between these two hypotheses.

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