

Characterization of the DNA Binding Region Recognized by Dihydrofolate Reductase from *Lactobacillus casei**

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Two specific DNA binding sites for the enzyme dihydrofolate reductase from *Lactobacillus casei* have been located by means of an immunoprecipitation assay within a 2900-base pair *L. casei* DNA fragment containing the *L. casei* dihydrofolate reductase structural gene, which was previously cloned into pBR322. The inserted *L. casei* DNA was mapped using restriction endonucleases, and the location and orientation of the structural gene coding for *L. casei* dihydrofolate reductase were determined. The two specific binding sites map at the 5' end of the structural gene, approximately 100 base pairs upstream from the start of the coding region.

Dihydrofolate reductase is of central importance in cell metabolism since it catalyzes the reduction of dihydrofolic to tetrahydrofolic acid which is required for the *de novo* synthesis of thymidine, purines, and glycine (Blakely, 1967). Tetrahydrofolate in turn is converted into a wide variety of 1-carbon group-transferring cofactors. Dihydrofolate reductase itself is the target for a group of clinically useful drugs such as trimethoprim and methotrexate which are widely used in the treatment of bacterial and protozoal infections and of neoplastic disease and as an immunosuppressant (Bertino and Johns, 1972).

Mechanisms by which cells become resistant to antifolate drugs have been studied in some detail, and resistant phenotypes are associated either with the overproduction of dihydrofolate reductase (Sheldon and Brenner, 1976; Sirotiak and Hachtel, 1969; Alt *et al.*, 1978; Smith *et al.*, 1982) or with the synthesis of a modified enzyme which has a lowered affinity for the drug (Sheldon and Brenner, 1976; Smith *et al.*, 1982).

Genetic evidence from *Diplococcus pneumoniae* (Sirotiak and Hachtel, 1969; Sirotiak and McCuen, 1973) suggested that autoregulation of dihydrofolate reductase plays a role in this organism. In order to test this hypothesis for *Lactobacillus casei* dihydrofolate reductase, we cloned the *L. casei* gene coding for dihydrofolate reductase into the multicopy plasmid vector pBR322, yielding the plasmid pWDLcB1 which contains a 2.9-kilobase pair insert of *L. casei* DNA comprising the dihydrofolate reductase structural gene and adjacent sequences and confers trimethoprim and methotrexate resistance to a sensitive host (Davies and Gronenborn, 1982). DNA binding properties of purified dihydrofolate reductase towards double-stranded linear and supercoiled DNA of pBR322 and pWDLcB1 were demonstrated (Gronenborn and Davies, 1981;

Gronenborn *et al.*, 1981). It was shown that *L. casei* dihydrofolate reductase binds specifically to a site on the DNA of pWDLcB1 with an association constant K_S of $3.66 \times 10^6 \text{ M}^{-1}$ as well as nonspecifically to pBR322 DNA with an association constant K_N of $5.09 \times 10^2 \text{ M}^{-1}$ in a highly cooperative manner (Clore *et al.*, 1982).

To further characterize the site of interaction between dihydrofolate reductase and its specific DNA binding site, we have mapped the inserted *L. casei* DNA in pWDLcB1 using restriction digests and localized the structural gene. Using the McKay immunoprecipitation assay (McKay, 1981), we demonstrate that two fragments of a *Hpa*II digest of the *L. casei* insert are specifically retained following complex formation with dihydrofolate reductase and subsequent antibody binding and that these fragments are located at the 5' end of the nucleotide sequence coding for the protein.

MATERIALS AND METHODS

Chemicals and Buffers—Restriction endonucleases were purchased from Boehringer Mannheim and New England Biolabs, and [γ - ^{32}P]ATP (3000 Ci/mmol) from Amersham Corp. Alkaline phosphatase and T4 polynucleotide kinase were obtained from Boehringer Mannheim and New England Biolabs, respectively. Other chemicals were of the highest purity commercially available and were used without further purification.

Protein Purification—Dihydrofolate reductase was purified as described by Dann *et al.* (1976), and its concentration was determined by assaying its catalytic activity.

DNA Purification—Plasmid DNA was prepared by a modification of the procedure of Clewell and Helinski (1959). DNA fragments were analyzed after digestion with restriction endonucleases (as recommended by the manufacturer) by agarose-gel electrophoresis and purified by extraction from low melting agarose gels that were run in TBE buffer (100 mM Tris, 100 mM boric acid, 5 mM EDTA, pH 8.3). The slice of agarose containing the desired restriction fragment was melted at 65°C and extracted twice with phenol (saturated with 0.3 M sodium acetate, pH 4.8) at 45°C. Further extraction with CHCl_3 and subsequent ethanol precipitation yield the purified DNA fragment in sufficient quantity (60–80% of input).

Fragments were dephosphorylated with alkaline phosphatase and radioactively end-labeled using [γ - ^{32}P]ATP and T4 polynucleotide kinase (Maxam and Gilbert, 1980).

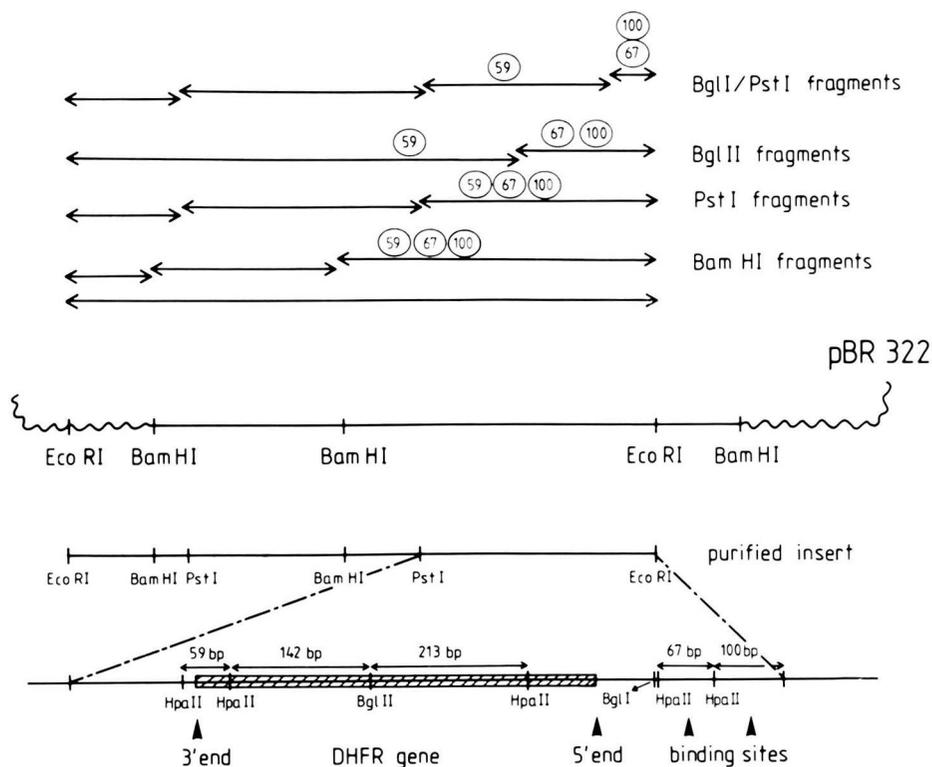
Antibody Production—1 mg of pure *L. casei* dihydrofolate reductase in Freund's complete adjuvant was injected intramuscularly into female adult rabbits at several sites. Two further booster immunizations were carried out after 10 and 20 days. After 28 days, serum samples were taken weekly.

DNA Binding and Immunoprecipitation—Binding of dihydrofolate reductase to DNA was performed in 20 mM Tris, pH 7.5, 100 mM NaCl, 2 mM dithioerythritol, 1 mM EDTA, 0.01% bovine serum albumin, 0.05% Nonidet P-40 in a total volume of 1 ml. After incubation at room temperature for 1 h, 50 μl of serum were added, and the mixture was incubated for a further hour at room temperature. The DNA-dihydrofolate reductase-antibody complex was immunoprecipitated with 50 μl of washed formalin-fixed *Staphylococcus aureus* (5 mg) for 20 min at room temperature. To reduce background binding, the precipitate was washed three times with 0.5 ml of wash

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FIG. 1. Structure of the plasmid pWDLcB1 containing the *L. casei* dihydrofolate reductase gene. Only the *L. casei* insert DNA and, in particular, the region around the structural gene are shown in detail. The restriction digests that were used for the blotting experiments are indicated in the top of the figure. (59), (67), and (100) represent the 59-, 67-, and 100-bp *Hpa*II fragments used as hybridization probes. They are drawn next to the fragment they hybridized to. The *Hpa*II fragments that were localized within the *Eco*RI insert are marked in the expanded lower part of the figure. Also shown is the orientation of the dihydrofolate reductase (*DHFR*) binding sites with respect to the structural gene.



buffer containing 50 mM Tris, pH 7.5, 350 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40, 0.01% bovine serum albumin. The DNA was eluted from the immune complex in 200 μ l of 2% sodium dodecyl sulfate in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0); and the supernatant was phenol-extracted three times and CHCl_3 -extracted once, and the DNA was ethanol-precipitated. The immunoprecipitated DNA fragments were separated on 2.5% agarose gels, and the dried gels were autoradiographed at -70°C with intensifying screens for 1–14 days.

Southern Blots—The DNA was fractionated on 2% agarose gels run in TBE buffer. Before blotting, the gel was washed twice with distilled water and soaked for 30 min in 0.5 M NaOH, 3 M NaCl. Blotting was carried out in $20 \times \text{SSC}$ ($1 \times \text{SSC}$: 15 mM sodium citrate, 150 mM NaCl, pH 7.0) overnight onto nitrocellulose paper (Schleicher & Schuell, BA85). Subsequent hybridization was carried out at 41°C overnight using standard procedures (Southern, 1975), and the dried filters were autoradiographed at -70°C with intensifying screens for 1–14 days.

RESULTS

In order to locate the specific binding site for dihydrofolate reductase on the *L. casei* DNA insert of pWDLcB1, it was necessary to cut the insert into smaller fragments, which were then used in a binding assay. Since the insert itself contains a *Bam*HI site, it was not possible to purify it as a single *Bam*HI fragment, although the *L. casei* DNA was inserted into the *Bam*HI site of pBR322. We therefore chose an *Eco*RI digest to purify the insert DNA since the *Eco*RI site on pBR322 is close to the *Bam*HI site, thus adding only 375 bp¹ of pBR322 DNA to the end of the cloned *L. casei* DNA. In addition, the single *Eco*RI site on the insert is located only 400 bp away from the distant *Bam*HI site, thus leading to only a small loss of inserted *L. casei* DNA within the purified *Eco*RI fragment. Fig. 1 shows a restriction map of the insert region of pWDLcB1 marking the *Bam*HI and *Eco*RI sites.

For the immunoprecipitation experiments, the purified *Eco*RI fragment, comprising most of the inserted *L. casei* DNA, was digested with the restriction endonuclease *Hpa*II, which yielded the most convenient set of small fragments

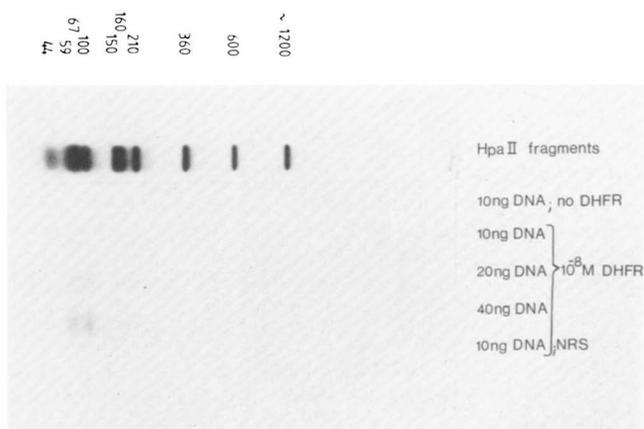


FIG. 2. Immunoprecipitation of *Hpa*II fragments from the purified *L. casei* insert DNA with a fixed concentration of dihydrofolate reductase (*DHFR*) (10^{-8} M) and varying amounts of DNA. Two controls were employed, one omitting the protein (no *DHFR*) and the other using normal rabbit serum (*NRS*). See "Materials and Methods" for experimental details.

with respect to their number and size distribution, namely 10 fragments of 44, 59, 67, 100, 150, 160, 210, 360, and 600 bp and ~ 1.2 kilobase pairs.

The immunoprecipitation assay is based on the ability to separate DNA to which at least one protein molecule is bound from free DNA by immunoprecipitation with an antibody raised against the DNA binding protein. Since radioactively labeled DNA is used in the binding experiments, fragments can be easily detected by separation on agarose gels with subsequent autoradiography.

Fig. 2 shows the result of an immunoprecipitation experiment using increasing concentrations of DNA, keeping the concentration of dihydrofolate reductase constant at 10^{-8} M. The first lane shows the size pattern of all 10 *Hpa*II fragments. While the two control samples (second lane: no dihydrofolate reductase (no *DHFR*); sixth lane: normal rabbit serum (*NRS*)) show no retention of fragments, samples that contained in-

¹ The abbreviation used is: bp, base pairs.

creasing amounts of DNA in the binding experiments show an increasing intensity for only two fragments of 67 and 100 bp, thus demonstrating specific DNA binding by dihydrofolate reductase to only these two fragments. Increasing the concentration of dihydrofolate reductase (up to 10^{-6} M) in the binding experiments (data not shown) results in increased retention of these two fragments in the immunoprecipitate, while only a slight increase in the intensity for the larger fragments is observed, confirming that the preferred retention of the 67- and 100-bp fragments is due to specific binding of dihydrofolate reductase to sites located within these fragments.

Localization of the specifically retained 67- and 100-bp fragments within the cloned *L. casei* DNA was achieved by Southern blotting (Southern, 1975). The end-labeled fragments were purified and hybridized to nitrocellulose filters to which restriction digests of the insert DNA from pWDLcB1 had been transferred. Blots were carried out with the two fragments that were specifically retained by dihydrofolate reductase in the immunoprecipitation assay, as well as with the 59-bp fragment which in part contains the nucleotide sequence coding for the COOH-terminal end of dihydrofolate reductase as revealed by DNA sequencing.² Fig. 3 shows two such blots. Using the 50-bp piece as a probe leads to the localization of this fragment within the large *Bam*HI fragment and the large *Bgl*II fragment of the *Eco*RI insert, while the 67-bp fragment is localized in the large *Bam*HI fragment and the small *Bgl*II fragment. The second specific binding fragment was blotted in the same way and also localized in the small *Bgl*II fragment within the large *Bam*HI fragment of the *Eco*RI insert (see Fig. 1).

We used the available amino acid sequence of *L. casei* dihydrofolate reductase (Bitar *et al.*, 1977) to search for possible restriction sites within the nucleotide sequence and compared these with the restriction pattern obtained from the *L. casei* insert in pWDLcB1. With the help of a unique possible *Bgl*II site within the predicted coding sequence and the location of a unique *Bgl*II cut within the *L. casei* insert DNA, we were able to determine the orientation of the dihydrofolate reductase gene in the cloned DNA. The arrangement of the gene within the insert is shown in Fig. 1 (this was confirmed by sequencing the relevant parts of the insert).^{3,4}

The localization of the 100-bp fragment to the end of the *Eco*RI fragment was achieved by end labeling the purified small *Bgl*II/*Eco*RI fragment of the insert DNA and subsequent cutting with *Hpa*II. Autoradiography showed two fragments of 190 and 100 bp in length. Since there is no possible *Hpa*II site 100 bp away from the *Bgl*II site within the DNA coding sequence predicted from the *L. casei* dihydrofolate reductase amino acid sequence (Bitar *et al.*, 1977), and since the Southern blotting located this fragment within the small *Bgl*II/*Eco*RI fragment (*i.e.* within the last 160 bp proximal to the *Eco*RI end of the insert), it has to be the end fragment of the insert. Thus, the two fragments that are bound specifically by *L. casei* dihydrofolate reductase are located upstream of the 5' end of the structural gene coding for dihydrofolate reductase.

Based on a length of the protein of 162 amino acids (Bitar *et al.*, 1977), the nucleotide sequence coding for dihydrofolate reductase has to be at least 486 bp long, thus positioning the two specifically bound fragments at least 100 bp upstream of the 5' end of the gene.

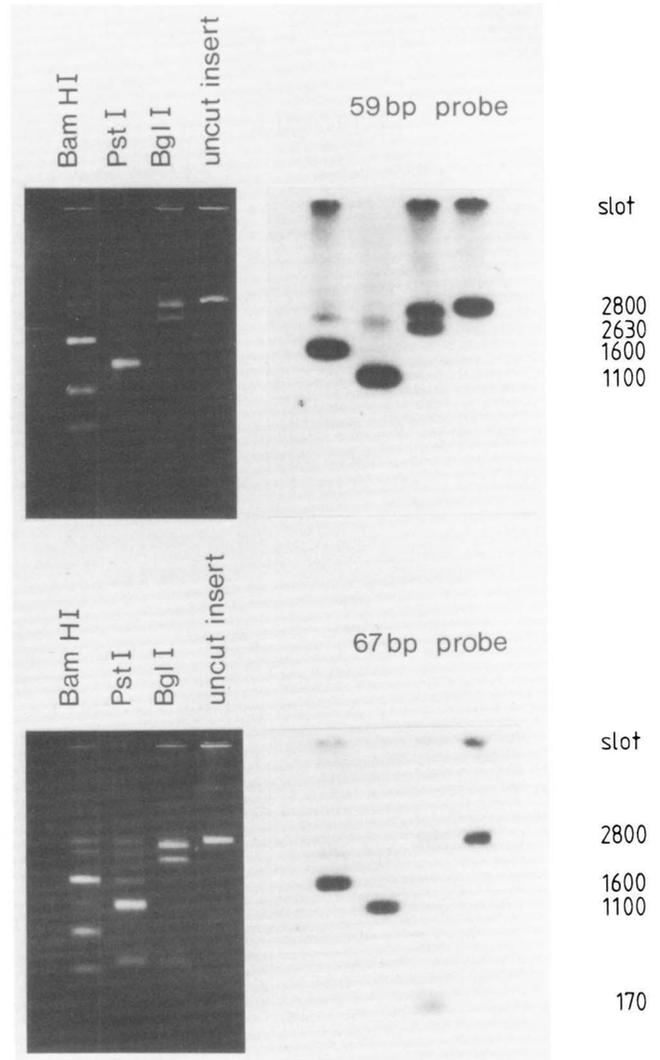


FIG. 3. Southern blotting with the 59-bp probe containing the coding sequence for the COOH-terminal end of dihydrofolate reductase and with the 67-bp probe which is one of the two fragments specifically retained by dihydrofolate reductase in the immunoprecipitation assay. See "Materials and Methods" for experimental details.

DISCUSSION

The results presented here show that dihydrofolate reductase from *L. casei* binds specifically to two DNA target sites located approximately 100 bp upstream of the 5' end of the structural gene and support the idea that specific binding of dihydrofolate reductase to DNA might be of physiological relevance to the regulation of bacterial dihydrofolate reductase *in vivo*. The available genetic data for *D. pneumoniae* and *Escherichia coli* in which mutants resistant to either trimethoprim or methotrexate result in increased mRNA synthesis and map in or near the structural gene (Sirotiak and Hachtel, 1969; Sirotiak and McCuen, 1973; Sheldon and Brenner, 1976) strengthen such a hypothesis. Although, in the case of *E. coli*, several of the trimethoprim-resistant mutants that overproduce dihydrofolate reductase were found to have mutations in the -35 region of the *fol* promoter (Smith and Calvo, 1982) and may thus be characterized as "promotor up" mutants, others had no detectable sequence alterations in the *fol* promoter or structural gene (Smith *et al.*, 1982). However, some aberrations in sequencing gels in a region 110 bp upstream from the *fol* promoter consisting of

² O. Kalderon, unpublished results.

³ R. W. Davies, A. M. Gronenborn, and G. M. Clore, unpublished results.

⁴ B. Gronenborn, A. M. Gronenborn, and G. M. Clore, unpublished results.

extraneous bands occurring in the A + G track superimposed over a wild type nucleotide sequence have been reported (Smith *et al.*, 1982) which, in the light of our binding experiments, might be of relevance for the increased amounts of dihydrofolate reductase found in such mutants.

A possible model for the regulation of dihydrofolate reductase synthesis which can account for the data so far available involves autogenous regulation with dihydrofolate reductase binding to sequences approximately 100 bp upstream of the 5' end of the structural gene, thus preventing transcription. A mutation in this site(s) should therefore result in a decreased affinity of the protein for the altered DNA target site, thus leading to an increase in the intracellular concentration of dihydrofolate reductase. It might seem surprising to find a possible binding site for a protein acting as a repressor at such a long distance from the start of the coding sequence, quite in contrast to the well characterized *lac* system (Gilbert, 1976), where the *lac* repressor protein binds to the 3' end of the promoter region only ~10 bp upstream of the *z*-gene. But the arrangement found in the *lac* system might not apply in a general way to all regulatory systems. For example, in the regulatory system of the phage λ , the binding sites for the λ -repressor are located at the 5' end of the promoter (Ptashne *et al.*, 1980), blocking a region immediately before the beginning of the messenger, covering the Pribnow box (Pribnow, 1975). Furthermore, these systems involve regulation by a special repressor protein and thus do not represent an autogenous regulatory system. In the case of SV40 large T antigen, for instance, which is involved in the regulation of its own synthesis (Hansen *et al.*, 1981; Myers *et al.*, 1981), the three DNA binding sites for the protein cover a stretch from 30 to 120 bp upstream from the coding sequence (Tooze, 1980). Furthermore, we do not know at present where the mRNA start is located in the *L. casei* case since there is no obvious Pribnow box within 30 nucleotides upstream of the 5' end of the coding sequence⁵ and it might well be that the mRNA has a long 5' leader sequence (a sequence very similar to an *E. coli* Pribnow box was, however, detected on the 67-bp *HpaII* fragment that was specifically retained by dihydrofolate reductase in the immunoprecipitation assay).

We therefore propose that the binding of *L. casei* dihydrofolate reductase to two specific sites at the 5' end of its structural gene is the underlying feature for the autogenous regulation of dihydrofolate reductase synthesis in *L. casei*.

⁵ R. W. Davies, unpublished results.

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