Expression of *Mycobacterium leprae* genes from a *Streptococcus mutans* promoter in *Escherichia coli* K-12

(expression vector/citrate synthase/minicells/cloning)

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ABSTRACT Genomic libraries of *Mycobacterium leprae* DNA partially digested with *Pst* I were constructed in the expression vector pYA626, which contains the promoter region from the *Streptococcus mutans* gene encoding aspartate β-semialdehyde dehydrogenase, which is very efficiently expressed in *Escherichia coli*. We have detected several clones that complement a mutation in the citrate synthase gene of *E. coli*. Southern blot analysis demonstrated that the complementing DNA was *M. leprae* DNA. Sodium dodecyl sulfate/polyacrylamide gel analysis of polypeptides produced by minicells containing the citrate synthase-complementing recombinant molecules demonstrated the production of a 46-kDa polypeptide. When the citrate synthase-complementing fragment was cloned in pYA626 in the reverse orientation, the recombinant molecule was no longer able to complement the mutation in the citrate synthase gene and no longer produced the 46-kDa polypeptide. When the DNA fragment was cloned in the *Pst* I site of pHCT79, so as to allow expression from the β-lactamase promoter, the resulting recombinant failed to complement the mutation in the *E. coli* citrate synthase gene yet still produced the 46-kDa polypeptide, but in one-fourth the amounts than when expressed from the *S. mutans* asd promoter. This demonstrates that *M. leprae* translational sequences can be recognized by *E. coli* translational machinery. Promoter expression vectors can be used to obtain expression of protein antigens to be used for early diagnosis of leprosy or components of a vaccine and proteins that are targets of potential anti-leprosy drugs.

Leprosy, an age-old chronic disease with a wide spectrum of manifestations, including gross skin disfigurement and peripheral nerve loss, afflicts over 15 million people in the world today (1). Its causative agent, *Mycobacterium leprae*, was shown to be associated with the disease by Gerhard Armauer Hansen in the early 1870s (2). Even so, *M. leprae* has been extremely difficult to study because of its inability to be cultivated in the laboratory. In the early 1960s, Shepard successfully cultivated *M. leprae* in the footpads of mice (3). Significant quantities of the organism became available for research upon the discovery that *M. leprae* produced a systemic infection in the nine-banded armadillo, *Dasypus novemcinctus* (4, 5).

We had previously screened genomic libraries of *M. leprae* DNA cloned in both plasmid and cosmids vectors and had not observed any complementation of a variety of mutations in amino acid, purine, and vitamin biosynthetic pathways or carbohydrate catabolic pathways in *Escherichia coli* K-12 (6). We cloned *M. leprae* DNA in the expression vector pYA626 and were able to demonstrate the expression of *M. leprae* polypeptides in minicells containing recombinant *M. leprae* molecules (6). In this manuscript, we describe the complementation of a mutation in the citrate synthase (EC 4.1.3.7) gene of *E. coli* K-12 by cloned *M. leprae* DNA that is expressed from the *asd* promoter of pYA626.

MATERIALS AND METHODS

Bacterial Strains and Methods. Table 1 lists and describes the *E. coli* strains used in this study. Phage P1 transduction (11), cosmid transduction (6), and transformation (12) were performed as described previously.

Media. *E. coli* strains were grown in L broth (13) supplemented with diaminopimelic acid and thymidine, if necessary, or minimal salts broth or agar supplemented with amino acids, nucleotides, vitamins, and glucose (11). L-Glutamic acid was sterilized by filtration.

Preparation of DNA. *M. leprae*, *Mycobacterium vaccae*, *Mycobacterium lllum*, and *D. novemcinctus* (nine-banded armadillo) DNAs were isolated and purified as described previously (6). Plasmid DNA was extracted by the Birnboim technique (14) with subsequent purification by centrifugation on cesium chloride/ethidium bromide density gradients, if necessary. DNAs were analyzed on 0.7% agarose gels (ref. 15, p. 150).

Enzymes. Restriction enzymes and DNA-modifying enzymes were obtained from Bethesda Research Laboratories, New England Biolabs, or Promega Biotech (Madison, WI) and used according to the supplier’s recommendations. Calf intestine alkaline phosphatase was obtained from Sigma.

Minicell Analysis. Minicells from 100-ml overnight cultures of χ925 and χ2338 containing various recombinant molecules were isolated and proteins were radiolabeled and visualized as described previously (6).

Hybridization Analysis. Southern hybridization analyses were performed with nick-translated *M. leprae* insert DNAs as probes as described previously (6). Colony hybridization was performed as described by Maniatis et al. (ref. 15, pp. 312–319).

Growth Assays. Overnight cultures of three different *gltA* (glutamate-requiring) mutants (χ2338, W620, and K2-1-4) with or without pYA1036 grown in L broth at 37°C were diluted 1:50 into 2.5 ml of glucose minimal medium containing necessary supplements and L-glutamic acid at 0, 10, 20, 30, 50, 100, or 200 μg/ml. After 11 hr of growth with aeration at 37°C, the individual cultures were subcultured as above into fresh medium containing the same amount of glutamic acid and incubated as above, and optical density was measured at 600 nm at various times after inoculation on a Spectronic 20 (Bausch and Lomb).

Abbreviations: bp, base pair(s); kb, kilobase(s).

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RESULTS

Cloning of an M. leprae Gene That Complements a Mutant in the Citrate Synthase Gene of E. coli. The expression vector pYA626 contains a 209-base-pair (bp) EcoRI–Pst I fragment from the asd gene of Streptococcus mutans that replaces the 755-bp EcoRI–Pst I fragment of pBR322. The S. mutans fragment contains the promoter region, the Shine–Dalgarno sequence, the portion of the asd sequence encoding the 41 amino-terminal amino acids of aspartate β-semialdehyde dehydrogenase (this sequence is in phase with that encoding 104 amino acids of the carboxyl terminus of β-lactamase), and the unique Pst I site downstream from the asd promoter (G. Cardineau and R.C., unpublished data). M. leprae DNA that had been partially digested with Pst I was size-fractionated on sucrose gradients. Fractions containing molecules of average size of 3 and 6 kilobases (kb) were ligated to pYA626 that had been digested with Pst I and treated with alkaline phosphatase. The resulting ligation mixture was used to transform an E. coli host strain with several auxotrophic mutations (χ2338). Over 5000 tetracycline-resistant transformants were obtained from each ligation and were subsequently pooled as individual libraries. Each library was diluted and tested for complementation of mutations in citrate synthase (gltA), dehydroquinase synthase (aroB), thymidylate synthase (thyA), succinyl-diaminopimelate aminotransferase (dapD), and tryptophan biosynthetic (trpBC) genes of χ2338. Complementation of the gltA16 allele from each library was observed at a frequency of 10⁻⁴ and that of the aroB mutation, at a frequency of 10⁻⁷. We did not observe complementation of any of the other mutations in this screening. Retransformation of χ2338 with plasmids isolated from clones selected for their ability to grow on minimal medium lacking glutamate in the presence of tetracycline showed 100% cotransformation of tetracycline resistance and the gltA-complementing activity. The clones that complemented the gltA mutation were able to form colonies 0.5–1.0 mm in diameter on minimal medium lacking glutamate and containing tetracycline after 4 days of growth at 37°C. Similar results were obtained when the clones were grown at 30°C. On minimal medium containing glutamate and tetracycline, χ2338 harboring pYA1036 (the recombinant molecule complementing the citrate synthase gene from the 3-kb library) formed colonies of similar size after 36–48 hr of incubation at 30°C or 37°C. An isogenic gltA⁺ derivative of χ2338 also formed colonies 0.5–1.0 mm in diameter after 36–48 hr of incubation at 30°C or 37°C. Digestion of pYA1036 by the restriction endonuclease Pst I followed by agarose gel electrophoresis revealed a single 2.6-kb insert in pYA626 (Fig. 1, lane 1). The gltA-complementing recombinant molecule found in the 6-kb library (pYA1037) also contained a 2.6-kb Pst I fragment, plus four additional small Pst I fragments (Fig. 1, lane 2). The presence of identically sized BamHI and EcoRV–XhoI fragments in both pYA1036 and pYA1037 showed that the position and orientation of the common 2.6-kb fragment was identical with respect to the asd promoter in the two plasmids (Fig. 1).

Southern blot analysis demonstrated that the cloned gltA-complementing DNA fragment hybridized very strongly to one fragment of M. leprae chromosomal DNA, less well to another fragment of M. leprae DNA, and weakly to unique Pst I fragments of the two other mycobacterial DNAs (Fig. 2). The weaker hybridization of pYA1036 to the lower band of M. leprae chromosomal DNA is probably due to the presence of an additional sequence in the M. leprae genome that is partially homologous to the gltA-complementing fragment, since hybridization of pYA1036 to the lower band was not observed until the autoradiograph was exposed for the longer period (20 hr) required to demonstrate the weak hybridization between pYA1036 and M. vaccae and M. "lufu" DNAs. Overexposure of the autoradiograph did reveal a very weak hybridization to E. coli DNA but no hybridization with the armadillo DNA (data not shown).

We screened the previously described pH79: M. leprae DNA cosmid libraries (6) in χ2819 for the presence of sequences that hybridize with the 2.6-kb gltA-complementing fragment by colony hybridization. Strong hybridization with 20 out of 1600 colonies was observed, thus confirming the presence of the gltA-complementing fragment in our original libraries, although we had not observed complementation of the gltA mutation in χ2338, probably because the M. leprae promoter for this gene does not function well in E. coli.

Expression of the gltA-Complementing Activity. To test whether the gltA-complementing activity was being expressed from the asd promoter of pYA626 or from its own promoter, we recloned the 2.6-kb gltA-complementing fragment in pYA626 and then screened for recombinant molecules containing the fragment in either orientation with respect to the asd promoter. The recombinant molecule pYA1040, which had the 2.6-kb fragment in the same orientation as in pYA1036, was able to complement the gltA16
Fig. 2. Southern hybridization of the 2.6-kb gltA-complementing DNA fragment to various chromosomal DNAs. (Left) Ethidium bromide-stained 0.7% agarose gel in which 1-µg samples of various chromosomal DNAs totally digested with Pst I were electrophoresed and transferred to GeneScreen (New England Nuclear). (Right) Autoradiogram of the blotted chromosomal DNA probed with the 2.6-kb Pst I gltA-complementing DNA fragment that had been labeled by nick-translation with 32P. Lanes: M, HindIII-digested λ DNA; 1, D. novemcinctus chromosomal DNA; 2, E. coli K-12 DNA; 3, M. leprae DNA; 4, M. vaccae DNA; 5, M. "lufu" DNA.

mutation in χ2338. However, when the 2.6-kb insert was cloned in the opposite orientation with respect to the asd promoter to yield pYA1041, no complementation of the mutation in the citrate synthase gene was observed (Fig. 3).

To determine whether any polypeptides were being synthesized by the cloned DNA, radiolabeled polypeptides produced in minicells containing various recombinant plasmids were analyzed. The vector, pYA626, specified the production of a 14-kDa fusion polypeptide consisting of the amino terminus of aspartate β-semialdehyde dehydrogenase and the carboxyl terminus of β-lactamase, as well as the 34-kDa tetracycline-resistance gene product (Fig. 4, lane 2). The two original gltA-complementing clones (pYA1036 and pYA1037) as well as the reconstructed gltA-complementing clone (pYA1040) all produce a unique polypeptide of 46 kDa that is not produced in minicells containing pYA1041 (Fig. 4, lanes 3, 4, 5, and 6). In fact, pYA1041 specifies the production of two different polypeptides of 14 and 25 kDa (Fig. 4, lane 5). Neither of these polypeptides is specified by any of the gltA-complementing recombinant molecules.

To test whether the 46-kDa gltA-complementing polypeptide was a fusion polypeptide with the amino terminus of the aspartate β-semialdehyde dehydrogenase, the 2.6-kb Pst I fragment was inserted into the Pst I site of pHC79, a small (6-kb) cosmid vector in which the β-lactamase promoter is located upstream from the Pst I site (16). Recombinant molecules containing the 2.6-kb insert in either orientation with respect to the β-lactamase promoter were analyzed. Neither pYA1044 (same orientation of the 2.6-kb insert as in the gltA-complementing molecules with respect to the β-lactamase promoter) nor pYA1045 was able to complement the gltA mutation in χ2338 (Fig. 3). If the gltA-complementing polypeptide was fused with the 41-residue amino terminus of the asd gene product, it should also form a fusion polypeptide with the 159-residue amino terminus of β-lactamase in pHC79 and produce a much larger fusion polypeptide (17). Both pYA1036 and pYA1044 specified the production of a 46-kDa polypeptide (Fig. 5, lanes 3 and 5). However, four times more of the 46-kDa protein was produced from the asd promoter than from the β-lactamase promoter when normalized to the 34-kDa tetracycline-resistance gene product.

Growth Measurements of gltA Mutants Containing pYA-

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Fig. 3. Schematic representation of recombinant molecules containing the gltA-complementing M. leprae DNA fragment. The orientations of the 2.6-kb gltA-complementing fragment from the two original gltA-complementing recombinant molecules are displayed with respect to the asd promoter, which would lie to the left of these maps. The 2.6-kb Pst I gltA-complementing M. leprae DNA fragment was recloned in the Pst I site of pYA626 or pHC79. The schematic representation of the fragment displays its orientation with respect to the asd or β-lactamase (bla) promoter. The recombinant plasmids were used to transform χ2338, and tetracycline-resistant transformants were screened for their ability to complement the gltA mutation. Glt⁺ and Glt⁻, glutamate-independent and -dependent, respectively.
mutations were host and complements derivatives grew cultures failed when plasmidless ml, per densities optical experiments isogenic gltA+ glutamate reached of X2338 g/ml. concentrations of and synthase X2338. which 6, pYA1041; 1036. containing pYA626; 3, containing fluorograph x925 minicells (Nuclear), dried, and the lysates were electrophoresed on a NaDodSO4/7.5–15.0% polyacrylamide gel. The gel was treated with ENHANCE (New England Nuclear), dried, and exposed to x-ray film; the resulting fluorograph is shown. Lanes: M, 3H-labeled protein standards; 1, χ925 minicells containing no recombinant molecule; 2, χ925 minicells containing pYA626; 3, χ925 minicells containing pYA1036; 4, χ925 minicells containing pYA1040; 5, χ925 minicells containing pYA1041; 6, χ925 minicells containing pYA1037.

**DISCUSSION**

The results demonstrate the expression of an *M. leprae* gene that partially complements mutations in three different alleles of the citrate synthase gene in *E. coli* K-12 when cloned in the proper orientation downstream from the very efficient *S. mutans* *asd* promoter. The complementing DNA fragment specifies a 46-kDa polypeptide when expressed from both the β-lactamase of pH79 and the *asd* promoter of pYA626. Thus, the 46-kDa *M. leprae* gene product is not a fusion polypeptide with the amino terminus of either β-lactamase or aspartate β-semialdehyde dehydrogenase. Therefore, the *M. leprae* translational initiation signal for this gene is recognized by *E. coli* translational machinery.

Failure to observe complementation of the citrate synthase mutation by the cloned *M. leprae* gene when the gene is expressed from the β-lactamase promoter, which resulted in a production of the 46-kDa polypeptide of one-fourth compared to when the *M. leprae* gene was expressed from the *asd* promoter, results in the reversion of all or some of the alleles. Therefore, the ability of χ2338 possessing pYA1036 to grow in minimal media lacking glutamate or with suboptimal concentrations of glutamate must be due to the presence of a functional citrate synthase on the cloned fragment.

**Stability of the gltA-Complementing Polypeptide.** The *lon* gene specifies a protease that degrades abnormal proteins (18), including fusions of β-galactosidase with foreign gene products (19). However, pulse-chase analyses of the *gltA*-complementing polypeptide produced in minicells from two isogenic *E. coli* strains differing only in the presence of *lon* or *lon*-9 alleles revealed no difference in the rates of degradation of the *M. leprae* protein in these two strains (data not shown).

**Fig. 4.** Fluorograph of polypeptides produced in minicells containing gltA-complementing and noncomplementing recombinant molecules. Minicells were isolated from χ925 that had been transformed with various pYA626: *M. leprae* recombinant molecules. The isolated minicells were labeled with [35S]methionine and lysed, and the lysates were electrophoresed on a NaDodSO4/7.5–15.0% polyacrylamide gel. The gel was treated with ENHANCE (New England Nuclear), dried, and exposed to x-ray film; the resulting fluorograph is shown. Lanes: M, 3H-labeled protein standards; 1, χ925 minicells containing no recombinant molecule; 2, χ925 minicells containing pYA626; 3, χ925 minicells containing pYA1036; 4, χ925 minicells containing pYA1040; 5, χ925 minicells containing pYA1041; 6, χ925 minicells containing pYA1037.

**Fig. 5.** Expression of the gltA-complementing polypeptide from the *asd* or *bla* promoters. Minicells were isolated from χ2338 clones containing various recombinant molecules, labeled with [35S]methionine, and lysed, and the lysates were electrophoresed on a NaDodSO4/7.5–15.0% polyacrylamide gel and fluorographed. Lanes: 1, χ2338 minicells containing pYA626; 2, χ2338 minicells containing pH79; 3, χ2338 minicells containing pYA1036; 4, χ2338 minicells containing pYA1041; 5, χ2338 minicells containing pYA1044; 6, χ2338 minicells containing pYA1045. CS, citrate synthase; Tc, tetracycline-resistance gene product.

1036. Growth rates were determined for W620, K2-1,4, and χ2338, which contain three different mutations in the citrate synthase gene of *E. coli* (the gltA6, gltA9, and gltA19 alleles), with and without pYA1036 in liquid minimal media with concentrations of l-glutamic acid varying from 10 to 200 μg/ml. χ2338 failed to grow in the absence of glutamate, whereas χ2338 containing pYA1036 in minimal media without glutamate reached an optical density one-sixth the level of cultures of χ2338 containing 200 μg/ml or cultures of an isogenic gltA strain after 48 hr of growth at 37°C. In repeated experiments with media containing 10 or 25 μg of glutamate per ml, cultures of χ2338 containing pYA1036 always reached optical densities that were 15 to 20 times higher than the plasmidless strain. In similar experiments using the gltA6 and gltA9 mutant strains with and without pYA1036, both strains failed to grow in liquid minimal media that lacked glutamate, even when they contained pYA1036. However, in repeated experiments, cultures of these strains harboring pYA1036 grew to densities that were 0.3 to 9 times higher than the plasmidless derivatives in media containing 10 or 25 μg of glutamate per ml. Thus, the cloned *M. leprae* gene partially complements the gltA6 and gltA9 mutations in these *E. coli* host strains.

Spontaneous reversion frequencies for the three gltA mutations were as follows: gltA6, <7.5 × 10−11; gltA9, <4 × 10−11; and gltA19, <5 × 10−11. Ultraviolet irradiation had no effect on the reversion frequencies of any of these alleles. Therefore, the ability of χ2338 possessing pYA1036 to grow in minimal media lacking glutamate or with suboptimal concentrations of glutamate must be due to the presence of a functional citrate synthase on the cloned fragment.

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Stability of the gltA-Complementing Polypeptide. The *lon* gene specifies a protease that degrades abnormal proteins (18), including fusions of β-galactosidase with foreign gene products (19). However, pulse-chase analyses of the gltA-complementing polypeptide produced in minicells from two isogenic *E. coli* strains differing only in the presence of *lon* or *lon*-9 alleles revealed no difference in the rates of degradation of the *M. leprae* protein in these two strains (data not shown).
promoter, indicates that decreased activity due to a lower level of expression precludes complementation on solid or in liquid minimal media. Even expression of the 46-kDa polypeptide from the asd promoter is insufficient for growth of X2338 to the level of an isogenic gltA+ strain. The observations that the presence of the recombinant plasmid, pYA1036, in strains W620 (which contains the gltA6 allele) and K2-1-4 (which contains the gltA9 allele) is inadequate to support growth of those strains in the absence of glutamate, yet enhances the growth of the strains in media containing suboptimal concentrations of glutamate suggest that the level of activity of the complementing gene product is a result of lower expression in these two strains. Alternatively, since W620 and K2-1-4 have shorter generation times than X2338, they may require greater citrate synthase activity per unit time to sustain their growth.

A mutation in the citrate synthase gene of E. coli results in auxotrophy for the glutamate family of amino acids (20, 21). Since the presence of the cloned M. leprae gene enhances the growth of three E. coli strains with three different gltA alleles, this strongly suggests that the cloned gene product is citrate synthase. The citrate synthase gene of Rickettsia prowazekii was identified by its ability to complement the gltA6 allele (22). The gltA6 allele has been mapped by P1 transduction to the known site of the citrate synthase gene, and strains possessing the gltA6 allele have been shown to be devoid of citrate synthase activity (unpublished results). Wheeler has isolated low citrate synthase activity in extracts of M. leprae cells grown in armadillos, although high levels of oxaloacetate were required for in vitro activity of the M. leprae citrate synthase (23). This result suggests that the M. leprae enzyme is different from the E. coli citrate synthase and could account for low in vivo activity in the recombinant clones.

The molecular weight of the cloned M. leprae gene product is 46,000, determined by NaDodSO4/polyacrylamide gel electrophoresis (PAGE). This is very similar to the molecular weight of the cloned E. coli citrate synthase, which was also determined to be 46,000 by NaDodSO4/PAGE (24), although the predicted molecular weight of the E. coli citrate synthase inferred from the DNA sequence is 48,069 (25). The molecular weight of the M. leprae citrate synthase from M. leprae cell extracts has not yet been determined.

The demonstration of the presence of the citrate synthase-complementing sequence in cosmid clones that were unable to complement the citrate synthase mutation and the inability to detect complementation of nine other auxotrophic mutations in E. coli host strains by recombinant cosmid molecules (ref. 6; this paper) imply that M. leprae promoters are not expressed or are very inefficiently expressed in E. coli. On the basis of the low level of expression of the 46-kDa polypeptide in minicells containing pYA1036 compared to the level of expression of the asd-β-lactamase fusion polypeptide (Fig. 5, lanes 2 and 3), it would appear that initiation of translation of the M. leprae citrate synthase gene is much less efficient than translation initiation of the asd-β-lactamase fusion polypeptide. In this regard, the Shine-Dalgarno sequence of the asd promoter has 8 of 9 bases complementary to the 3′ end of the 16S ribosomal RNA of E. coli (G. Cardineau and R.C., unpublished data). Thus, expression of other mycobacterial genes may be enhanced by constructing protein or operon fusions. We have inserted a DNA sequence with multiple cloning sites downstream from the asd promoter in both plasmid and λ vectors to facilitate such constructions.

The ability to express M. leprae genes in E. coli by using promoter expression vectors will provide many new opportunities to understand M. leprae and the disease of leprosy. Expression of protein antigens of M. leprae should permit identification of those entities that are important for protective immunity against leprosy and ultimately provide a source of material for a possible vaccine. Young et al. have recently identified several polypeptide antigens by screening an Agt11::M. leprae library with monoclonal antibodies directed against specific M. leprae antigens (26). The ability to complement a mutation in E. coli demonstrates that a functional M. leprae enzyme has been expressed. Expression of other M. leprae enzymes that are putative targets of anti-leprosy drugs will provide material to screen the potential effectiveness of such drugs in E. coli in lieu of costly screening in mouse footpad assays (27) and will facilitate analysis of mechanisms of action of such drugs.

We especially want to thank Charles Shepard for his encouragement and for supplying the infected armadillo livers. Charles Shepard died on February 18, 1985. We dedicate this manuscript to him.

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