1985

Construction And Analyses Of Genomic Libraries Of Mycobacterium Leprae Dna (Cloning, Cosmids, In Vivo Packaging, Citrate Synthase, Minicells).

William Robert Jacobs
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CONSTRUCTION AND ANALYSES OF GENOMIC LIBRARIES OF MYCOBACTERIUM LEPRAE DNA

The University of Alabama at Birmingham PH.D. 1985

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CONSTRUCTION AND ANALYSES OF GENOMIC LIBRARIES OF *MYCOBACTERIUM LEPRAE* DNA

by

WILLIAM ROBERT JACOBS

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in the Department of Microbiology
in The Graduate School, University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

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ABSTRACT OF DISSERTATION
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Degree Ph.D. Major Subject Microbiology

Name of Candidate William Robert Jacobs

Title Construction and analyses of genomic libraries of *Mycobacterium leprae*

The goal of the research presented in this dissertation was to construct genomic libraries of *Mycobacterium leprae*, the causative agent of leprosy, and to obtain expression of functional *M. leprae* proteins in *Escherichia coli* K-12. Genomic libraries were initially constructed in cosmid vectors. A set of strains of *E. coli* K-12 were constructed that permitted amplification of *in vitro* packaged recombinant cosmid transducing particles through *in vivo* repackaging of recombinant cosmid molecules. Thermal induction of these thermoinducible, excision-defective lysogens containing recombinant cosmid molecules yield high titers of packaged recombinant cosmids, low titers of plaque-forming units, and permit the storage of recombinant cosmid libraries as phage lysates. The utility of these strains for complementation analyses, *in situ* assays for enzymatic activities, and expression analysis were demonstrated for *Mycobacterium leprae*, *Mycobacterium vaccae*, *Mycobacterium* "lufu", *Salmonella typhimurium*, and *Streptococcus mutans* cosmid genomic libraries. Complementation of auxotrophic mutations or mutations in carbohydrate catabolic pathways in *E. coli* by mycobacterial DNA cloned into cosmid or plasmid vectors was not observed. Genomic libraries of *M. leprae* DNA partially digested with *Pst*I were subsequently constructed in the

expression vector pYA626, which contains the promoter region from the S. mutans genes encoding aspartate β-semialdehyde dehydrogenase which is very efficiently expressed in E. 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. SDS-polyacrylamide gel analysis of polypeptides produced by minicells containing the citrate synthase-complementing recombinant molecules demonstrated the production of a 46kDa polypeptide. The 46kDa polypeptide was not to a fusion polypeptide with the amino terminus of the aspartate semialdehyde dehydrogenase, suggesting that M. leprae translational signals are being recognized by E. coli ribosomes. The ability to express functional M. leprae proteins in E. coli provides a novel method for study of the uncultivatible micro-organism, M. leprae.

Abstract Approved by: Committee Chairman Roy Curtiss III

Program Director Richard J. Goergen

Date 5-31-85 Dean of Graduate School R. J. Boozer

DEDICATION

Charles Shepard was the first person to demonstrate the multiplication of Mycobacterium leprae cells in the laboratory. In 1960, he was able to observe multiplication of the organism in experimentally infected mouse footpads. As a result, for the first time in history, researchers had a system to study experimentally the interactions of M. leprae with a host. This system has been used to determine the sensitivities of M. leprae to various drugs, and to study the immunological response of a host to M. leprae. Charles made many significant scientific contributions, not only to leprosy research but to other areas as well: he was one of the researchers who identified and cultivated the causative agent of Legionnaires' disease, Legionella pnuemophila.

On February 18, 1985, Charles Shepard died of a heart attack while swimming at Emory University. His death saddened his many friends, family, and fellow leprosy researchers around the world. Charles had recognized the potential benefits of applying recombinant DNA technology to the study of M. leprae and initially contacted Roy to engage our lab in leprosy work. Charles supplied the infected armadillo tissue for our work, as well as excellent insights and unfailing enthusiasm. I had the great privilege of having Charles Shepard serve on my doctoral dissertation committee the four years prior to his death and as a result had spent time with him on numerous

occasions. I was often impressed not only by his broad knowledge, but also by his sincere desire to continually broaden the scope of his knowledge to enhance study of leprosy. I have fond memories of sharing dinners with Charles in which the conversation would cover a wide array of topics and reveal both his caring and often times humorous nature. We in our group certainly miss Charles and I dedicate this dissertation to him.

ACKNOWLEDGEMENTS

It has been almost six years since I started my quest for a Doctor of Philosophy degree and in that time I have truly been blessed with help and encouragement from many individuals towards that pursuit. There are too many individuals to mention that taught, challenged, and molded me in my efforts to learn bacterial genetics and molecular biology. I have a great sense of appreciation and respect for those with whom I've had such interactions.

I've had the great privilege of having a husband and wife team, Roy Curtiss III and Josephine E. Clark-Curtiss, as co-advisors directing my doctoral research and providing a productive and stimulating research enviroment in which to work. Josie spent considerable time in the lab teaching me techniques of bacterial genetics and sharing my joys and frustrations in my research efforts. We regularly exchanged our thoughts and ideas on daily experiments and I greatly appreciate her support, encouragement, and interaction. I greatly appreciate Roy's advice and encouragement to me in my growth as a scientist. Both his breadth of knowledge and his wisdom to know what was really relevant to my goals makes him a very fine advisor. I have to admit that he still knows a lot more genetics than I do and he has set standards of excellence that I will endeavor to reach. Both Josie and Roy suffered through the editing of my terrible writing. I've learned a lot and thank them both for their excellent critiques of my work.

We've had excellent technical help in our research efforts. Lynn Ritchie spent cosiderable time developing the procedure of isolation of Mycobacterium leprae cells from armadillo liver in our lab. The procedure is very laborious, but essential to my work. Lynn was a lot of fun to work with and I thank her for her help. Martin Docherty, the Flying Scotsman, performed many of the minicell experiments described in this dissertation in collaboration with me. He's a gifted researcher and I sincerely thank him for his efforts and friendship.

I've worked many long days since I started this work, and my wife, Lyn, has patiently and lovingly endured this entire experience. She deserves a lot of credit, and I thank her whole-heartedly for her love and support.

I also wish to thank Gayle Knapp, Charles Turnbough, and Frank Griffin for their careful reading of my dissertation and their valuable comments.

Almost two years ago, we moved from the University of Alabama at Birmingham to Washington University in St. Louis. Both institutions have provided excellent resources for my work. I owe a special thanks to the Washington University Computing Facility for providing an excellent means for the processing of this dissertation and I thank Amy Papian for typing those tables that I could not get the computer to print. My sincere thanks is also extended to the Graduate School of the University of Alabama at Birmingham for their excellent help in the preparation of this dissertation.

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INTRODUCTION

Leprosy is an age-old plague of mankind that still afflicts over 15 million people today, primarily in developing countries in tropical areas of the world (1). The causative agent of leprosy, Mycobacterium leprae, was identified microscopically in biopsy material from patients with leprosy in 1870, making it the oldest known etiological agent of disease in man (3). Leprosy is a chronic granulomatous disease with a wide range of clinical manifestations including skin disfigurement and peripheral nerve loss. There are five common histopathological classifications of the different forms of the disease (8). These classifications range from two polar extremes called tuberculoid and lepromatous leprosy and a borderline form that lies in between. Lepromatous leprosy is characterized by the presence of huge numbers of bacteria accumulated in infected tissues and a continuous bacteremic state. Patients with tuberculoid leprosy have only a few bacteria localized in the skin and nerves. The immunolgical responses are considerably different between the two forms as the lepromatous patients lack a cellular immune response to M. leprae antigens (see review,9). In contrast, tuberculoid patients have a normal or even excessive cellular immune response which may account for the increase of nerve damage found in this form. The borderline form of leprosy is an unstable state and frequently progresses to one of the two polar forms.

Prior to the early 1940's, there was no effective therapy for treating victims of leprosy, and consequently patient isolation was the only method of control. Faget et. al reported a marked improvement in leprosy patients when treated with glucosulfone, a drug that had been used sucessfully in treatment of guinea pigs that had been experimentally infected with Mycobacterium tuberculosis (2). The parent compound, 4,4'-diaminodiphenylsulfone (dapsone), was determined to be safe, inexpensive, and extremely effective against M. leprae, making it the drug of choice for treating leprosy. However, dapsone-resistance has been reported in an increasing number of cases each year, and thus poses a serious problem in the control of the disease (7). Rifampicin and clofazamine are also used in the treatment of leprosy, but their cost and adverse side effects prevent wide use in developing nations (4).

M. leprae is an obligate intracellular parasite that seems to multiply in the cytoplasm and phagolysosomes of macrophages (6). Cultivation of M. leprae on any media has thus far proved to be impossible. The first successful experimental laboratory infection came in 1960 when Charles Shepard demonstrated the multiplication of M. leprae in mouse footpads (10). Significant quantities of M. leprae cells became available for researchers when the armadillo, whose natural body temperature is 30 to 33^{o}C, was found able to develop a systemic infection when experimentally inoculated with M. leprae (5,12). The generation time of M. leprae has been estimated to be 11 to 13 days in the mouse footpad (11), and a two-year

incubation time is required from initial infection of an armadillo until the time of harvest of the bacteria (13).

Recombinant DNA technology offers tremendous promise for the study of the enigmatic organism M. leprae. Expression of M. leprae genes in Escherichia coli would provide a readily obtainable source of M. leprae proteins such as antigens or enzymes. The well defined genetic system of E. coli could be used to study the genetics and mechanisms of gene regulation of M. leprae, thus providing a better understanding of M. leprae itself. This dissertation describes: (i) the construction of M. leprae genomic libraries using a cosmid vector, (ii) the construction of E. coli strains that permit the repackaging of recombinant cosmid molecules in vivo thereby allowing amplification of packaged recombinant cosmid molecules and facilitating screening of cosmid libraries, (iii) the construction of genomic libraries of M. leprae in a plasmid expression vector, which allowed expression of M. leprae DNA in E. coli, and (iv) the expression of a functional M. leprae enzyme activity that complements a mutation in the citrate synthase gene of E. coli.

This dissertation has as its body three manuscripts. The first and the third manuscripts describe work that was primarily done by me. The second manuscript was written by J.E. Clark-Curtiss and includes the construction of M. leprae and M. vaccae cosmid libraries and libraries using the expression vector pYA626 which were my responsibilities.

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IN VIVO REPACKAGING OF RECOMBINANT COSMID MOLECULES FOR ANALYSES of SALMONELLA TYPHIMURIUM, STREPTOCOCCUS MUTANS AND MYCOBACTERIAL GENOMIC LIBRARIES

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ABSTRACT

Strains of Escherichia coli K-12 have been constructed that permit the amplification of in vitro-packaged recombinant cosmid transducing particles through in vivo repackaging of recombinant cosmid molecules. Thermal induction of these thermoinducible, excision-defective lysogens containing recombinant cosmid molecules yields high titers of packaged recombinant cosmids and low levels of plaque forming units. These strains permit the storage of cosmid libraries as phage lysates. These strains are used to amplify packaged recombinant cosmid libraries of Mycobacterium leprae, Mycobacterium vaccae, Salmonella typhimurium, and Streptococcus mutans DNA. The utility of these strains for complementation analysis, in situ assays for enzyme activities, and expression analysis by transducing UV-irradiated cells with high titer lysates of individual repackaged recombinant cosmids is demonstrated. The construction of contiguous and noncontiguous libraries was compared for the successful identification of cloned genes. Construction of noncontiguous libraries allows the dissociation of desired genes from genes that are deleterious to the survival of a cosmid recombinant and (ii) permits the cloning and selection for two unlinked traits to result in a selected phenotype. The use of in vivo repackaging of recombinant cosmids permits: (i) the amplification of the original in vitro packaged collection of transducing particles, (ii) the storage of cosmid libraries as phage lysates, (iii) facilitated complementation screening, (iv) expression analysis of UV-irradiated cells by repackaged recombinant cosmids, (v)

in situ enzyme or immunolgical screening, and (vi) facilitated recovery of recombinant cosmid molecules containing transposon inserts.

INTRODUCTION

The technique of cosmid cloning (11) has provided an efficient means of cloning segments of DNA as large as 45 kb (30). Cosmids are plasmid cloning vectors containing the cohesive end site (*cos*) of bacteriophage λ. The recombinant DNA molecule generated by ligating foreign DNA between two linearized cosmid molecules can be packaged *in vitro* if the distance between the two *cos* sites is 38 to 52 kb (11). Transduction of *Escherichia coli* K-12 by these lambdoid particles results in the introduction of large recombinant molecules which are maintained within the cell as covalently closed circular plasmids.

Our group is interested in studying *Mycobacterium leprae* by constructing libraries of the genomic DNA and taking advantage of the genetic systems of *E. coli* for analyses of the cloned DNA. The amount of DNA available from the uncultivatible microorganism *M. leprae* is limited because significant quantities of the *M. leprae* cells can only be obtained from infected armadillos after a two year incubation and a tedious isolation procedure (10). Therfore, amplification of constructed *M. leprae* libraries is most desirable. Analyses of recombinant DNA libraries for expression of of functional genetic traits is facilitated by transferring the recombinant molecules from the original recipient host to other *E. coli* strains with a variety of mutations. Recombinant cosmids are not efficiently transferred by transformation because of their large size. Construction of mobilizable cosmid vehicles permits transfer with the help of a conjugative plasmid (21), yet it is not permissable under

the NIH guidelines to clone DNA from an organism that does not normally exchange DNA with E. coli into an E. coli host possessing a conjugative plasmid (45). Another approach for the transfer of recombinant cosmid molecules is to repackage them into bacteriophage lambda particles. Several groups have reported that plasmid molecules containing a lambda cos site could be packaged in vivo with the help of an exogenously added helper phage (19,22,41,51,53,54). The number of infectious particles in such lysates limited their use. One way to circumvent this problem is by use of lysogens of E. coli K-12 that contain an integrated prophage that is defective in normal excision function (29,37, W.R. Jacobs, J.E. Clark-Curtiss, L.R. Ritchie, and R. Curtiss III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H147, p.130). We have consructed a set of thermoinducible lysogens of E. coli K-12 containing a prophage that is defective in both excision and recombination functions. When these strains containing recombinant cosmid molecules are induced, they yield high titers of transducing particles containing the recombinant cosmid molecules and low titers of infectious phage. The original cosmid library is thus amplified as a phage lysate of transducing particles. We had previously described the construction of and amplification of an M. leprae cosmid library (10). In this manuscript, we further describe the construction of additional in vivo cosmid packaging strains and demonstrate their usefulness in the analyses of cosmid libraries prepared on M. leprae, Mycobacterium vaccae, Salmonella typhimurium, and Streptococcus mutans DNAs.

MATERIALS AND METHODS

Bacterial strains, phage, media, and diluents. The bacterial strains used in this study are listed in Table 1. λ cI857 b2 redβ3 S7, λ hφ80 Δatt c, and λ b2 c were obtained from Nat Sternberg. λ cI857 b2 redβ3 was isolated as a spontaneous revertant of λ cI1857 b2 redβ3 S7 on SC180 (suppressor-free E. coli K-12 strain from O. Reyes). λ vir came from this laboratory. The strains were grown in TYM broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.4% maltose) prior to infection by λ or cosmid transducing particles and L broth (36) or superbroth (38) for amplification of recombinant clones, plasmid isolation and conjugations. Complex media were supplemented with diaminopimelic acid at 100 μg/ml for strains χ2001, χ2341, and χ2367 and thymidine (THD) at 40 μg/ml for all strains with a thyA mutation. Minimal salts broth or minimal salts agar (12) was supplemented with 0.5% carbohydrates; amino acids, nucleotides and vitamins were supplemented as previously described (13). EMBO agar (25) was made with EMB agar base (Difco Laboratories, Detroit, Mich.) to which 0.5% yeast extract, 0.5% NaCl, and 10 mM $MgSO_4$ were added. Buffered saline with gelatin (BSG,[12]) and TMGS (10 mM Tris, pH 7.4, 10 mM $MgSO_4$, 0.1% gelatin, and 100 mM NaCl) were used as diluents for bacteria and lambdoid particles, respectively.

Construction of lysogens. Bacteriophage λ lysates and P1L4 lysates were prepared; transductions and conjugations were performed as described previously (6,12,13). Transconjugants of the mating between χ2382 and WA802 were selected on minimal glucose agar plates

suppelemented with methionine, THD, and 20 μg of trimethoprim (Sigma Chemical Co., St. Louis, Mo.) per ml. Isolates which required thymidine for growth, indicative of the presence of the thyA mutation, were subsequently tested for the phenotypic properties of recA and relA mutations by sensitivity to ultraviolet light (7) and inability to grow on minimal glucose plates supplemented with THD at 40 μg/ml and serine, methionine and glycine at 100 μg/ml (52), respectively. Selection for rare λ b2 lysogens involved plating cells to which the λ b2 phage had been adsorbed along with 10^9 λ b2 c and 10^9 λ ηφ80 Δatt c on EMBO agar. Pseudolysogens give rise to irregularly-shaped purple colonies as a result of phage infection of λ-sensitive cells occurring during growth, while true lysogens appear as perfectly round, white, nonmucoid colonies (25) at a frequency of 10^{-5} to 10^{-6} with Rec^+ strains and 10^{-6} to 10^{-7} with recA strains. Putative lysogens were tested for thermosensitivity and the ability to be infected with λ vir at 30°C. Tandem polylysogens were distinguished from monolysogens by spotting a loopful of liquid culture grown at 30°C on LE392 cells and incubating at 42°C. Monolysogens of λ b2 phage excise at a very low frequency and will not result in lysis of the indicator LE392 cells whereas tandem polylysogens will.

Enzymes. Restriction endonucleases and T4-DNA ligase were purchased from New England Biolabs, Boston, Mass. or Bethesda Research Laboratories, Bethesda, Md. Deoxyribonuclease I (DNaseI) and calf intestine alkaline phosphatase were purchased from Sigma.

Preparation of DNA. Cosmids pHC79 (30) and pMMB34 (20), as well as recombinant cosmid molecules were isolated according to the technique of Birnboim and Doly (2), and further purified by cesium chloride-ethidium bromide gradient centrifugation purification, if necessary. DNA manipulations were carried out as described by Mantiatis et. al. (40). To prevent ligation of tandem pMMB34 vectors, right and left arms were prepared in a manner analagous to that described by Ish-Horowicz and Burke for pJB8 (32). pMMB34 was totally digested with either HpaI or HindIII; treated with alkaline phosphatase; extracted with phenol-chloroform; digested with BamHI and the right and left arms were separated on an agarose gel, electroeluted, and purified over a NACS column (Bethesda Research Laboratories). Chromosomal DNAs were isolated from M. vaccae and S. mutans 6715 (UAB66,[43]) as previously described (10,31). S. typhimurium LT2 DNA was a generous gift of Charles Turnbough.

Cosmid cloning. Preparation of packaging extracts from NS428 and NS433 (50) and in vitro packaging of ligated DNA were performed as previously described (42). M. vaccae DNA was partially digested with either BamHI or SalI to an average size of greater than 40 kb, mixed with pHC79 in a molar ratio of 2 to 1 (vector to insert), ligated and in vitro packaged. The in vitro packaged BamHI or SalI generated libraries were transduced into χ2367 and χ2764, respectively, and recombinant clones were selected on L agar containing 25 μg of ampicillin (Ap) per ml.

S. typhimurium DNA was partially digested with Sau3A and 25 μg of DNA were layered on a 4-ml 5 to 25% sucrose gradient which was

centrifuged in a SW56 rotor (Beckman Instruments, Palo Alto, Calif.) at 34,000 rpm for 10.5 h at 10°C. Fractions were collected by gravity flow, the DNA was precipitated with the addition of yeast carrier RNA, resuspended in water, and 1 μg of DNA of an average size of 35 kb was mixed with a 4-fold molar excess of each of the pMMB34 arms, ligated, and *in vitro* packaged. The resulting particles were transduced into χ2764 and recombinant clones were selected on L agar containing 50 μg of kanamycin (Km) per ml.

Twenty-five micrograms samples of *S. mutans* DNA, partially digested with *Sau*3A, *Eco*RI, or *Pst*I, were layered on separate 5.0-ml 10 to 40% sucrose gradients, centrifuged in a Beckman VTi65 rotor at 10°C for 105 min, and fractions were collected as described above. A library of DNA sequences contiguous within the *S. mutans* chromosome was consructed by ligating DNA fragments greater than 40 kb to *Pst*I-cut pHC79. Libraries of DNA fragments that are not normally contiguous (noncontiguous) within the *S. mutans* chromosome were generated in two ways. *Eco*RI-cleaved DNA fragments of 5 to 10 kb were religated to themselves at a concentration of greater than 200 μg/ml to regenerate noncontiguous high molecular weight DNA. This DNA was again partially digested with *Eco*RI and sized as above. DNA fragments of greater than 40 kb in size were ligated to *Eco*RI-cut pHC79. The other noncontiguous library was generated by mixing *Sau*3A-cleaved DNA fragments of 5 to 10 kb with *Bam*HI-cut pHC79. In all the *S. mutans* DNA ligations, pHC79 was added at a 4 to 1 molar ratio of vector to insert. Again, the ligated DNA fragments were *in vitro* packaged and the resulting particles were transduced into χ2819. Recombinant

clones were selected on L agar plates containing either 10 μg tetracycline (Tc) per ml for the PstI and EcoRI libraries or 25 μg Ap per ml for the Sau3A library. Incubation for growth on all selection plates was at 30°C.

Amplification of packaged recombinant cosmids. Cells to be transduced with packaged recombinant cosmids were grown in TYM broth to late log phase, pelleted, and suspended in TMGS with volume equal to the original volume. Transduction with in vitro packaged or in vivo packaged recombinant cosmids was performed by mixing 0.1 ml of packaged recombinant cosmids with 0.2 ml of prepared cells and allowing adsorption to occur by incubating at 30°C for 30 min. After adsorption, 0.7 ml of superbroth were added to the transducing particle-cell suspension and this was incubated for an additional 45 min at 30°C to allow expression of the antiobiotic resistance genes. Recombinant clones were selected for growth at 30°C on L agar or in L broth containing 25 μg of Ap per ml, 10 μg of Tc per ml or 50 μg of Km per ml, respectively. Amplification of individual recombinant clones or libraries was achieved by growing the clones or pools of clones in L broth containing 0.4% glucose and the appropiate antibiotic. Small scale in vivo packaged lysates were prepared in 250-ml baffled flasks containing 25 ml of media. Large scale in vivo packaged lysates were prepared in two-liter baffled flasks containing 500 ml of media. Individual recombinant clones or pools of recombinant clones were inoculated at an optical density at 600 nm of less than 0.05 and grown 30°C with moderate shaking to an optical density equal to 0.3. The prophage were induced by incubating the

flasks in a 45°C waterbath with occasional shaking followed by incubation with vigorous shaking for 1 to 5 h at 37°C. After the 37°C incubation, the cells were lysed by adding 0.01 volume chloroform and shaking an additional 10 min. The cell debris was removed by centrifuging the lysate in a Sorvall SS34 rotor at 10,000 rpm at 4°C for 10 min. The supernatant fluid was carefully decanted into small bottles to which a few drops of chloroform were added followed by vigorous shaking. These lysates were stored at 4°C.

High titer lysates of in vivo-packaged recombinant cosmids were prepared by incubating the two-liter flasks with vigorous aeration for 2.5 h at 37°C after the initial induction. The flasks containing induced cells were suspended into an ice water bath for 15 min. The induced, unlysed cells were pelleted by centrifugation at 4000×g for 10 min at 4°C. The pellets were suspended in 7.0 ml TMGS. The cells were lysed with chloroform resulting in a viscous suspension from the released cellular DNA. This suspension was incubated in the presence of DNase I (100 μg/ml) for 20 min at 37°C. The cell debris was pelleted by centrifuging the suspension in an SS34 rotor at 12,000 rpm for 10 min at 4°C. The packaged recombinant phage could be further purified by cesium chloride equilibrium density gradient centrifugation. Cesium chloride was added at a concentration of 0.709 g per g of lysate. The suspension was centrifuged in a SW41 rotor (Beckman) for 24 h at 30,000 rpm at 10°C. The repackaged recombinant cosmids could be visualized by light scattering in the center of the tube by shining a light from the top of the tube. The repackaged recombinant cosmids were collected with a 20 gauge needle and syringe,

and dialyzed extensively against TMGS. A yield of 1×10^{11} to 3×10^{11} transducing particles conferring antibiotic resistance was routinely obtained from a 500 ml preparation.

Complementation analysis. Repackaged recombinant cosmid molecules were transduced into cells of the particular test strain, prepared as above for λ infection, with at a multiplicity of infection of 0.1 in a total volume of 0.3 ml. After a 20 min adsorption period, 0.7 ml superbroth was added and the cells were incubated an additional 45 min to allow expression of the transduced genes. The cells were then diluted and spread on selective media.

Expression analysis. The protocol was similar to that used for phage infection of UV-irradiated cells (33). A uvrA mutation was introduced into χ2866 or χ2875 to preclude UV-repair and mutation in the lon gene was introduced into χ2875 to determine its effect on the stability of expressed foreign proteins. Twenty ml of cells were grown in minimal salts broth containing 0.4% maltose and all twenty amino acids at the previously described concentrations (10), in a 250-ml baffled flask to 10^8 cells per ml. The cells were pelleted at 8000 rpm in an Sorvall SS34 rotor for 10 min at room temperature. The pellet was suspended in 20 ml minimal salts broth with no supplements and irradiated with 1000 J/m^2 of UV light. The cells were pelleted as above and suspended in 1/10 volume growth media containing 10 mM $MgSO_4$, but without methionine. One ml of cells were transferred to an Eppendorf tube and incubated at 37^oC for 2 h to allow degradation of existing messenger RNA. One to five repackaged recombinant phage were added per cell and allowed to adsorb for 20 min prior to addition

of 10 μCi of [^{35}S]-methionine (1000 Ci/mole). After 10 min labelling, cells were lysed and analyzed as previously described on SDS-polyacrylamide gel electrophoresis (10).

In situ assays for identification of dextranase, protease, and glucosyltransferase activities. Repackaged recombinant cosmids were transduced into χ2831 at a multiplicity of infection of 0.1, incubated 45 min for expression of the antibiotic resistance gene, and then diluted and spread to a density of 200 to 300 antibiotic-resistant colonies per selective plate. The plates were incubated at 30°C 18 to 20 h so that the average colony size was 0.75 mm. To screen for dextranase or protease activities, recombinant clones were plated on L agar supplemented with THD and Ap or Tc. Colonies were gently overlayed with 12 to 14 ml of 0.05 M sodium acetate, (pH 5.4), 0.7% blue dextran, and 1.4% agar that had been sterilized by autoclaving and precooled to 55°C to screen for dextranase activity. To lyse the colonies by thermal induction of prophage, plates were incubated in an inverted position for 8 to 14 h at 42°C, and an additional 24 h at 37°C. The production of dextranase by a recombinant clone resulted in a zone of clearing in the blue agar overlay. In a similar fashion as for the dextranase screen, protease activity was detected by overlaying colonies with 12 to 14 ml of molten solution of 0.05 M sodium phosphate buffer (pH 6.8), 5% skim milk and 1.4% agar followed by thermal induction to induce lysis. Positive protease activity resulted in a zone of clearing in the normally opaque overlay. Glucosyltransferase activity was detected by providing a substrate (sucrose) and a primer (dextran

T-10) for enzymatic activity (a modification of a technique developed by R. Russell, personal communication). Recombinant clones were plated on L agar supplemented with THD, Ap or Tc, 0.4% sucrose and 0.2% dextran T-10. After sufficient growth, the colonies were incubated at 42°C for 8 to 14 h followed by an additional 48 to 72 h incubation at 25°C. A large, white, dome-like mucoid-like appearance was considered positive for glucosyltransferase activity. In all three cases, transducing particles conferring Ap^r or Tc^r and the observed activities were recoverable from the screened lysed colony.

RESULTS

Amplification of packaged recombinant cosmid molecules. Transduction of χ2367 with in vitro packaged recombinant cosmids generated from the BamHI-digested M. vaccae DNA yielded 250 recombinant clones. Four independent recombinant clones containing different pHC79::M. vaccae molecules, determined by restriction endonuclease digestion analyses on agarose gels, were used to analyze in vivo cosmid packaging in χ2367. Low titre lysates prepared by inducing individual clones of χ2367 containing pHC79::M. vaccae molecules transferred Ap-resistance to λ-sensitive cells at frequencies greater than 10^8 per ml of lysate (TABLE 2). These lysates contained no viable cells and treatment of these lysates with DNase I had no effect on transfer of Ap-resistance. When the λ-resistant strain χ1849 (27) was used as a recipient host, transfer of Ap-resistance was 3 to 4 logs less than transfer to λ-sensitive cells (data not shown). Restriction endonuclease analysis of plasmids isolated from χ2367 recombinant clones prior to in vivo packaging and from a mixture of thousands of cells obtained after transduction with the respective lysate showed identical DNA banding patterns (data not shown). We could thus conclude that transfer of the Ap-resistance was mediated by the in vivo packaged recombinant cosmids and that in vivo packaging had not altered the genetic structure of the recombinant molecules.

Effect of the recA mutation on in vivo repackaging of recombinant molecules. Monomeric circular molecules of bacteriophage λ

containing a single cos site are not packaged in vivo, but oligomers generated by recombination can be (16,18,49). Since the wild-type recA gene product mediates oligomer formation of various plasmids (1,28), a mutation in the recA gene might affect the yield of recombinant cosmid molecules that are packaged in vivo. To analyze such an effect, the recombinant cosmid pYA1010 was transduced into the isogenic recA$^+$ χ2763 and recA13 χ2674 strains and repackaged in these strains. The absence of the wildtype recA gene product did not signficantly affect the yields of repackaged recombinant cosmid molecule pYA1010 (TABLE 3). Restriction analyses of pYA1041 isolated as a plasmid before in vivo packaging and after transduction, again, showed identical banding patterns. The λ cI857 b2 redβ3 S7 prophage in these strains will excise at low frequencies and can be assayed by plating on a strain containing a tyrT (supF) mutation. There is no significant difference between the yields of plaque forming units from these lysates as result of the recA mutation (TABLE 3).

Complementation analyses A contiguous genomic library of S. typhimurium DNA was constructed by ligating right and left arms of pMMB34 to fragments of S. typhimurium DNA of 35 kb to 40 kb in size that had been cleaved with Sau3A. The resulting ligation was packaged in vitro, transduced into χ2764, and recombinant clones were selected on L agar plates containing Km. A total of 928 Km-resistant clones were used for further study. Patches of overnight growth of each of the 928 clones from a L agar plate containing Km were pooled and used to prepare an amplified library of repackaged recombinant cosmids. A diluted suspension of pooled cells was inoculated into 200 ml of LB

containing 0.4% glucose and 50 μg of Km per ml in a two-liter flask. The cells were grown and induced as described above. At 1 h intervals post induction, till 5 h; samples were removed for subsequent lysis with chloroform. The number of transducing particles conferring Km-resistance and the number of infectious phage were determined as described above (TABLE 4). The yield of packaged recombinant cosmids did not increase significantly after 2 h of induction and the number of plaque-forming units was consistently 4 to 5 logs lower than the number of packaged recombinant cosmids.

Each of the 928 Km-resistant clones was screened for the presence of recombinant molecules that complemented mutations in the ara, leu, pro, and gal mutations of the host. Based on insert sizes of 37 kb and assuming that there was no biasing against the cloning of any fragment of DNA, the library consisted of 99.98% of the S. typhimurium genome (8). Three to nine recombinant clones were found that complemented each of the four mutations screened for in the original library (TABLE 5). All three of the recombinant cosmids that complemented the ara mutation also complemented the leu mutation, consistent with their close proximity of less than 0.5 min on the S. typhimurium genetic linkage map (47). The amplified library of repackaged pMMB34::S. typhimurium molecules was screened for the number of recombinant cosmids that complemented the ara, leu, pro, gal, and xyl mutations in HB101. The frequencies of complementation of the previously screened genetic markers were all approximately one log lower than had been observed in the screening of the original library, with the exception of the ara complementing recombinant which

dropped two logs (TABLE 5). However, even with this drop, 10^4 recombinant cosmids that complemented the *ara* mutation were present per ml of amplified lysate.

To determine if *M. vaccae* DNA could complement mutations in *E. coli* a *M. vaccae* library was screened for complementation of 9 *E. coli* mutations. *M. vaccae* DNA that had been partially digested with *Sal*I was ligated to *Sal*I-cleaved pHC79, packaged *in vitro*, transduced into χ2764, and 650 Ap^r clones were selected. No complementation of the *ara*, *leu*, *pro*, and *gal* mutations of the host strain was observed. A significant fraction of the clones (280/650) were both Tc^r as well as Ap^r, indicative of tandem pHC79 cosmid molecules within the recombinant molecule. A reciprocal crossover event between two tandem vector molecules results in the formation of a vector and a recombinant molecule containing only a single vector molecule (32). Segregants containing just the vector have a selective advantage over clones containing recombinant molecules, thus explaining some of the instability problems of recombinant cosmid molecules (32). Since recombinant cosmid molecules containing tandem vectors are unstable, a pool of the 370 of the Ap^r, Tc^s recombinant cosmids were induced to prepare a lysate of repackaged recombinant cosmids. Complementation analyses of the *pro*, *thyA*, *trpE*, *ara*, *gal*, or *lac* mutations of χ2001 also revealed no complementation by the cloned DNA. Restriction endonuclease analyses of 10 random amplicillin-resistant transductants of χ2001 revealed a variety of cloned sequences present indicating that the library was diverse.

Further characterization of the pHC79::M. leprae libraries that were previously described (10) was done by performing complementation analyses by transducing χ2338 and screening for complementation of the dapD, gltA, ΔtrpBC, ΔthyA57, and aroB genetic markers. No complementation with the cloned M. leprae DNA was observed.

Infection of UV-irradiated E. coli cells with repackaged recombinant cosmids. Expression analysis of individual repackaged recombinant cosmid molecules was performed by using the isogenic uvrA χ2866 or the uvrA lon χ2875 strains. The lon gene encodes a protease that is known to degrade abnormal proteins of E. coli (5,24,48) as well as fusions of β-galactosidase fused foreign proteins (55). The products of genes of other organisms expressed in E. coli might be abnormal, and may be degraded by the lon gene product. Degradation of these proteins would decrease the likelihood of their being found in screens of recombinant libraries. A Tn10 insertion was isolated in the tsx gene of E. coli which yielded a 33% P1-mediated cotransduction frequency with proC. It was transduced into a strain containing the lon-9 allele (TABLE 1). The cotransduction frequency of tetracycline-resistance with the mucoid phenotype of lon-9 mutation is 11% allowing easy transfer of the lon mutation to other E. coli strains. χ2875 was consructed for expression analysis and χ2877 for in situ screening.

High titer lysates of three individual repackaged recombinant pHC79::M. leprae molecules were prepared for infection into UV-irradiated χ2866 or χ2875 cells. The UV-irradiation severely damages the host chromosomal DNA thereby preventing any de novo

synthesis of polypeptides(33). Repackaged recombinant cosmids were added at a multiplicity of 1 to 5 followed by labelling with [^{35}S]-methionine. The cells were lysed and polypeptides run on a SDS-polyacrylamide gel. A few polypeptides other than the tetracycline-resistance gene product were synthesized from two of the three recombinant molecules analyzed. The intensity of the band of one of the newly synthesized polypeptides was greater when analyzed in the strain containing the lon-9 mutation compared to the strain with the wild type lon gene. These recombinant molecules are being further characterized to determine if the synthesis is being directed from M. leprae transcription and tranlation signals and to see what role the lon protease plays in the stability of expressed M. leprae proteins.

in situ screening of amplified recombinant cosmid libraries. Various activities possibly relevant to the virulence of S. mutans, were screened in different recombinant cosmid libraries. Two types of libraries were constructed: contiguous and noncontiguous. Non-contiguous libraries were constructed for two reasons: (i) to enhance the cloning of genes encoding a desired activity that may be closely linked to another gene that, in high copy number, was deleterious to the survival of the recombinant molecule or (ii) to clone unlinked genes that are separated by distances larger than can be cloned in a cosmid and which are needed to give the selected phenotype. All three libraries were initially transduced into χ2819 selecting for the appropiate antibiotic resistance. The libraries were amplified by in vivo cosmid packaging and subsequently tranduced into χ2831, which contains a prophage that contains a normal S gene

permitting cell lysis to facilitate *in situ* screening (FIG. 1). Dextranase, glucosyltransferase, and protease activities were found among the 3 different libraries (TABLE 6). Dextranase-positive clones were found in all three libraries, with two distiguishable phenotypic types found in the *Sau*3A library. One type gave a much more pronounced zone of clearing of the blue dextranase as compared to the other type found in all three libraries. The glucosyltransferase and protease activities were only found in the two noncontiguous libraries.

DISCUSSION

We have constructed a set of thermoindicible, excision-defective lambda lysogens of E. coli K-12 that permit the repackaging of recombinant cosmid molecules into bacteriophage lambda transducing particles in vivo. Thermal induction of the these strains containing recombinant cosmid molecules as plasmids yields high titers of repackaged recombinant cosmid molecules. These strains are all lysogenized with a λ prophage whose genome can not excise efficiently from the E. coli chromosome. Induction of these strains containing recombinant cosmid molecules results in a lysate that contains only one plaque-forming phage for every 10^4 to 10^5 repackaged recombinant cosmid molecules. This repackaging of recombinant cosmid molecules does not depend on recombination systems of the host or phage, consistent with previous findings of other groups (19,22,37,41). In vivo packaging lysogens have been made and tested in three different genetic backgrounds. χ2367 was constructed as an EK2 host, but yields about a log fewer repackaged recombinant cosmids than than the HB101 and WA802 derivatives. The WA802 derivatives have the advantage over the HB101 derivatives, that they contain a hsdR mutation as opposed to the hsdS mutation. Both of the hosts are defective in host restriction activity, but the WA802 derivatives are proficient in host modification activity allowing for the modification of cloned DNA. Isogenic in vivo packaging strains differing at S gene of the prophage, which is a cell lysis protein, offer versatility in their use. χ2819, which contains a defective prophage S gene, facilitates

the preparation of high titer lysates of *in vivo* packaged recombinant cosmids; since the induced, unlysed cells containing *in vivo* packaged recombinant cosmids can be concentrated by centrifugation of the cells. χ2831, which contains a normal prophage *S* gene, facilitates screening of expressed cloned DNA; since cells are easily lysed permitting *in situ* screening of products present within the cell.

Cosmid cloning permits the cloning of large segments of DNA, which reduces the number of clones needed to represent a genome. Construction of genomic libraries provides the source of the genetic material to be analyzed. It is therefore important that the library represent the entire genome. In the case of *M. leprae*, in which the available amount of chromosomal DNA is limiting, the amplification of the genomic library provides a source of *M. leprae* DNA for additional library constructions. The amplification of any library of DNA sequences will most likely result in a change in the distribution of DNA sequences as a function of the generations of growth of the *E. coli* host into which the library was introduced. The repackaging of recombinant cosmid molecules *in vivo* does not differ in this regard, but is a useful extension of cosmid cloning because it allows amplification of *in vitro* packaged recombinant cosmid transducing particles. Cosmid libraries can then be stored as stable phage lysates as opposed to a collection of *E. coli* cells containing 50 kb recombinant plasmids which can undergo selective loss.

Cosmid libraries maintained as plasmids can be unstable for a variety of reasons, but many of these can be circumvented. The presence of two tandem vector molecules within a recombinant molecule

is quite unstable because the recombination product results in the formation of a vector and and a recombinant cosmid molecule containing a single vector molecule. Segregants containing only the vector will have a selective advantage in growth during the amplification of a library and will outgrow the clones containing recombinant molecules because they replicate faster (32). Such constructs can be avoided by preparing the vector in such a way to preclude the formation of such unstable molecules (32) and by fractionating the insert DNA on sucrose gradients prior to ligation with the vector.

Certain DNA sequences will be lost because of something intrinsic within the sequence; i.e., instability as a result of the inability to be replicated or instability as a result of a recombination event which could delete a sequence. The ability to _in vivo_ package in the absence of host and phage recombination systems minimizes the loss of sequences due homologous but not to non homologous to recombination.

Certain other sequences might be lost because they encode some gene product which when expressed, is deleterious to the growth of the _E. coli_ host. Other DNA sequences may confer a selective advantage because they promote the growth of the _E. coli_ and those cells will increase within the population. The precise nature of the instability of the _ara_ complementing clone of _S. typhimurium_ DNA library is unknown. The library was prepared in such a way to preclude tandem vector formation. Based on the above suppositions for DNA that is known to be expressed in _E. coli_, certain recombinant molecules within the _S. typhimurium_ library would decrease in the population while others should increase. The use of genomic libraries to

propagate a source of genetic material is favored in a situation in which the cloned DNA is not expressed in that host. This seems to be the case thus far for mycobacterial DNA. We have failed to observe complementatation of E. coli mutations by M. leprae, M. vaccae, and Mycobacterium "lufu" DNA cloned into the cosmid pHC79 (10). However, we have observed complementation of a mutation in the citrate synthase gene of E. coli when the M. leprae gene was expressed from an expression vector (manuscript in preparation), and so the inability of M. leprae promoters to be expressed by E. coli RNA polymerase is a likely explanation of our observations.

We constructed noncontiguous S. mutans libraries as well as contiguous libraries assuming that when cloning such large segments of DNA it was possible that genes of interest could be physically near other genes whose presence might be deleterious to the growth of the host. The inability to obtain a S. typhimurium recombinant cosmid that only complemented the ara mutation and not the leu mutation suggests that such a clone might be unstable. The construction of non contiguous libraries would physically dissociate existing linkages. Although not conclusive, we only found recombinant cosmids with glucosyltransferase and protease activities in the noncontiguous libraries. Phenotypes which require the interaction of two or more unlinked genes widely separated on a chromosome could not be found in contiguous libraries. The possibilities that the glucosyltransferase and protease activities require one or more gene products or that the genes encoding activity are linked to a gene that when expressed is deleterious to the E. coli are under investigation.

The ability to repackage recombinant cosmid molecules *in vivo* offers a variety of useful advantages for the screening of recombinant cosmid libraries. Complementation analysis of *E. coli* mutations by cosmid libraries is facilitated by such a system. Amplified cosmid libraries can be introduced efficiently into *E. coli* strains with a variety of mutations by simple transduction rather than transformation. Transformation efficiencies are strain-dependent and inefficient for plasmids as large as recombinant cosmids. Also, the majority of characterized mutations in *E. coli* are in strains that are not suitable hosts for the introduction of foreign DNA. Host strains for gene cloning have mutations that inactivate the host restriction systems that recognize foreign DNA. Use of *E. coli* host strains possessing a *hsdR* mutation allows introduction of foreign DNA without restriction and its modification so that amplified cosmid recombinants can be introduced into any restriction proficient *E. coli* host for complemetation analysis.

Preparation of a high titer lysates an *in vivo* packaged recombinant cosmid molecule, with low numbers of background helper phage permits the analysis of the polypeptides produced by a particular repackaged recombinant molecule by infection of UV-irradiated cells. This is an alternative to analyzing the expression of recombinant cosmids as plasmids. The maxicell technique of expression analysis is dependent upon the statistical probability that a plasmid is less likely to incure DNA damage than the chromosomal DNA (46). Molecules that do incure damage are degraded in the maxicell strain. Recombinant cosmid molecules, because of their large size, are more likely to suffer DNA

damage than a small plasmid molecule thus making the maxicell technique less useful for expression analysis of cosmids. Segregation of some plasmids into minicells is inefficient, but the reason is not clearly understood (9). Transduction of UV-irradiated cells therefore is a useful alternative for expression analysis. The use of this technique for expression analysis of M. leprae recombinant cosmids provided an efficient means of screening large segments of M. leprae DNA for its ability to be expressed in E. coli. The use of χ2866 and χ2875, which contain a wild type lon allele and the lon-9 allele, respectively, for analysis of expression recombinant cosmid DNA offers a means of testing the effect of the lon protease on the stability of foreign proteins.

Another use of in vivo packaging strains for analyses of cosmid libraries was demonstrated with the in situ screening for enzyme activities of the S. mutans libraries. This method allows for the lytic release of functional proteins expressed from recombinant cosmids at the same time that a proportion of those recombinant cosmid molecules are packaged into bacteriophage λ particles. Such particles can be directly recovered from the screened colony eliminating the need for replica plating of the screened library. This method is not only applicable to enzyme screening, but immunologicalscreening as well (17,31).

Use of these strains will also make the technique of transposon mutagenesis of recombinant cosmid molecules less burdensome. White et. al. demonstrated that a lysate made upon induction of λ cI857 lysogen containing a recombinant cosmid molecule and a transposon

inserted in the bacterial chromosome resulted in packaging of recombinant cosmid molecules into which the transposon had inserted (54). Transposon mutants in the recombinant cosmid molecule could be identified by transducing a susceptible host and selecting for growth on plates containing both the antibiotic resistances of the transposon as well as the cosmid. However, the large amount of infectious phage in their lysates hindered the effectiveness of this technique.

The virulence mechanisms of pathogenic organisms can be analyzed by the cloning of genes encoding virulence determinants into E. coli. Selection and subsequent analysis of such genes are facilitated by taking advantage of the well defined genetic system of E. coli. Cosmid cloning permits the cloning of large segments of DNA, therby reducing the number of clones necessary to reprersent a genome. The use of in vivo repackaging of recombinant cosmids is a useful extension of cosmid cloning as it permits: (i) the amplification of the original in vitro packaged collection of transducing particles, (ii) the storage of cosmid libraries as phage lysates, (iii) facilitated complementation screening, (iv) expression analysis of UV-irradiated cells by repackaged recombinant cosmids, (v) in situ enzyme or immunolgical screening, and (vi) facilitated recovery of recombinant cosmid molecules containing transposon inserts.

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TABLE 1. Bacterial strains

Strain	Genotype	Source
WA802	F⁻ lacY1 glnV44 galK2 galT22 λ⁻ metB1 hsdR2	34
HB101	F⁻ ara-14 leuB6 proA2 lacY1 glnV44 galK2 λ⁻ recA13 rpsL20 xyl-5 mtl-1 thi-1 hsdS20	4
RR1	F⁻ ara-14 leuB6 proA2 lacY1 glnV44 galK2 λ⁻ rpsL20 xyl-5 mtl-1 thi-1 hsdS20	3
LE392	F⁻ lacY1 glnV44 galK2 galT22 λ⁻ tyrT58 metB1 hsdR14 trpR55	Philip Leder[a]
χ2001	F⁻ ΔaraC766 fhuA53 dapD8 proA318 minA1 Δ69[gal-chlD] λ⁻ tyrT58 ΔgalU183 ΔtrpE5 minB2 rfb-2 recA56 relA1 ΔthyA57 endA1 oms-1 Δasd-4 rpoB402 cycB2 cycA1 hsdR2	9
χ2338	F⁻ ΔaraC766 fhuA53 dapD8 minA1 gltA16 Δ69[gal-chlD] λ⁻ ΔtrpBC13 minB2 rfb-2 gyrA25 ΔthyA57 endA1 oms-1 aroB15 cycB2 cycA1 hsdR2	17 steps from χ1776 (15)
χ2341	F⁻ ΔaraC766 fhuA53 dapD8 proA318 minA1 Δ69[gal-chlD] λ- ΔtrpBC13 minB2 rfb-2 gyrA25 ΔthyA57 endA1 oms-1 Δasd-4 cycB2 cycA1 hsdR2	9

TABLE 1 (Continued)

Strain	Genotype	Source
χ2367	χ2341 lysogenized with λ cI857 b2 redβ3 S7	This work
χ2382	HrfOR91 thr-16 tsx-63 purE41 glnV42 λ⁻ trpE63 his-53 recA56 relA1 ΔthyA57 tte-1 mtlA9 metE98 cycB2 cycA2 (O-lysA argA his ... serA F)	Isolated in 14 steps from χ540 (14)
χ2763	RR1 lysogenized with λ cI857 b2 redβ3 S7	this work
χ2764	HB101 lysogenized with λ cI857 b2 redβ3 S7	this work
χ2811	F⁻ lacY1 glnV44 galK2 galT22 λ⁻ ΔthyA57 metB1 hsdR2	χ2382 X WA802
χ2813	F⁻ lacY1 glnV44 galK2 galT22 λ⁻ recA57 ΔthyA57 metB1 hsdR2	χ2382 X WA802
χ2815	χ2811 lysogenized with λ cI857 b2 redβ3 S7	this work
χ2819	χ2813 lysogenized with λ cI857 b2 redβ3 S7	this work
χ2831	χ2813 lysogenized with λ cI857 b2 redβ3	this work
χ2844	F⁻ tsx-462::Tn10 λ⁻	Selected as a T6^r transductant of MG1655 (26) using λ561(35)

TABLE 1 (Continued)

Strain	Genotype	Source
χ2845	F⁻ ara-14 leu-6 azi-6 fhuA29 lacY1 tsx-462::Tn10 lon-9 purE42 glnV44 galK2 λ⁻ trpE38 lpcB rpsL73 xyl-5 mtl-1 thi-1	P1L4 (χ2844)→ RGC103 (23)
χ2865	F⁻ lacY1 glnV44 galK2 galT22 λ⁻ metB1 malE::Tn10 hsdR2	P1(TST1)→WA802 TST1 from T. Silhavy
χ2866	F⁻ lacY1 glnV44 galK2 galT22 λ⁻ metB1 uvrA1 hsdR2	P1(χ2660)→χ2865 Sel'd Gal⁺, UV^s
χ2867	F⁻ lacY1 tsx-462::Tn10 lon-9 glnV44 galK2 galT22 λ⁻ metB1 uvrA1 hsdR2	P1(χ2845) → χ2866
χ2869	WA802 lysogenized with λ cI857 b2 redβ3	this work
χ2873	F⁻ lacY1 tsx-462::Tn10 lon-9 glnV44 galK2 galT22 λ (cI857 b2 redβ3) metB1 hsdR2	P1L4 (χ2845) → χ2869

TABLE 1 (Continued)

Strain	Genotype	Source
χ2875	F⁻ lacY1 Δtsx-465 lon-9 glnV44 galK2 galT22 λ⁻ metB1 uvrA1 hsdR2	from χ2867[b]
χ2877	F⁻ lacY1 Δtsx-466 lon-9 glnV44 galK2 galT22 λ (cI857 b2 redβ3) metB1 hsdR2	from χ2869[b]

[a] LE392 is a calcium-resistant isolate of the strain ED8654 (44) that was isolated by L. Enquist (personal communication).

[b] selected as a fusaric acid resistant derivative (39).

TABLE 2. Yield of repackaged recombinant cosmid molecules from the *in vivo* packaging of pHC79::*M. vaccae* DNA recombinant molecules in χ2367.

Recombinant cosmid	Repackaged recombinant cosmids per ml lysate[a]	Yield of repackaged recombinant cosmids per Ap^r cell[b]
pYA1010	2.1×10^8	5
pYA1011	3.5×10^8	2
pYA1012	3.8×10^8	10
pYA1013	1.2×10^8	9

[a]Titered as the number of transducing particles conferring Ap-resistance using HB101 cells.

[b]Individual clones were grown to 5×10^7 to 1×10^8 cells per ml before thermal induction, induced at 45°C for 15 min, and incubated for 2 h at 37°C before lysis with chloroform.

TABLE 3. Effect of the recA allele on in vivo packaging of recombinant cosmid pYA1010.

Recombinant cosmid clone	Yield of repackaged recombinant cosmids per Ap^r cell[a]	Plaque-forming units per repackaged recombinant cosmid[b]
χ2763(pYA1010) recA+	51	3.6×10^{-4}
χ2764(pYA1010) recA	44	1.0×10^{-4}

[a]Individual clones were grown to 5×10^7 to 1×10^8 cells per ml, before thermal induction, induced at 45°C for 15 min, and incubated for 2 h at 37°C before lysis with chloroform. Repackaged recombinant cosmids were titered as the number of transducing particles conferring Ap-resistance using HB101 cells.

[b]Plaque-forming units were titered on LE392 cells.

TABLE 4. Yields of packaged recombinant cosmid molecules of an amplified pMMB34::*S. typhimurium* library as a function of time after induction in χ2764.

Time after induction (h)	Repackaged recombinant cosmids per ml of lysate[a]	Plaque-forming units per repackaged recombinant cosmid[b]
1	5.6×10^{8}	8.5×10^{-5}
2	9.2×10^{8}	2.2×10^{-4}
3	9.2×10^{8}	5.2×10^{-4}
4	9.2×10^{8}	6.3×10^{-4}
5	1.16×10^{9}	4.6×10^{-4}

[a]Pool of recombinant clones were grown to 1×10^{8} cells per ml, shifted to 45°C for 15 min, and then incubated at 37°C. Samples were taken at hour intervals, lysed with chloroform, and then the number of repackaged recombinant cosmids conferring Km-resistance were titered on HB101 cells.

[b]Plaque-forming units were titered on LE392 cells.

TABLE 5. Comparison of complementation frequencies of *E. coli* genetic markers of a pMMB34::*S. typhimurium* DNA library before and after amplification by *in vivo* cosmid packaging in χ2764.

Genetic Marker	Original Library[a]	Amplified Library[b]
ara	3×10^{-3}	3.2×10^{-5}
leu	9×10^{-3}	4.9×10^{-3}
pro	6×10^{-3}	7.5×10^{-4}
gal	3×10^{-3}	1.3×10^{-4}
xyl	ND[b]	1.5×10^{-3}

[a]Frequencies are the ratio of complementing recombinant cosmids in the original *in vitro* packaged library to the total number of recombinant cosmids conferring Km-resistance.

[b]Frequencies are the ratio of complementing recombinant cosmids in the amplified *in vivo* packaged library to the total number of recombinant cosmids conferring Km-resistance using HB101 cells. There were 9.2×10^8 repackaged recombinant cosmids per ml of lysate

[c]not determined

TABLE 6. Screening of *S. mutans* cosmid libraries for dextranase, protease, and glucosyltranserase activities.

Library	Number of original clones	Library Description	Dextranase	Glucosyl-transferase	Protease
PstI	1200	sized, contiguous	6/2100	0/4000	0/4000
EcoRI	1700	sized, noncontiguous	3/4200	3/2400	0/4300
Sau3A	$>10^4$	unsized, noncontiguous	13/2900	4/1200	3/4800

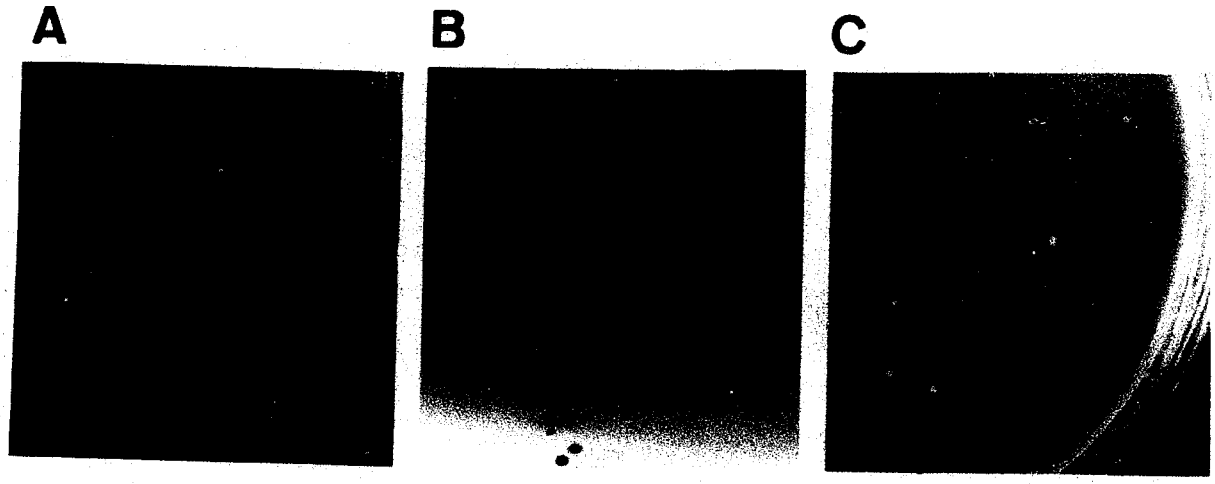

FIG.1. In situ screening assays for dextranase, protease, and glucosyltransferase activities. χ2831 was transduced with various S. mutans cosmid libraries, transductants were selected on antibiotic-selective media, and then various assays were performed as described in the Materials and methods. Examples of the dextranase (A), protease (B), and glucosyltransferase (C) screens are shown above.

MOLECULAR ANALYSIS OF THE DNA AND CONSTRUCTION OF GENOMIC LIBRARIES OF *MYCOBACTERIUM LEPRAE*

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ABSTRACT

Molecular analysis of DNA from Mycobacterium leprae, M. "lufu" and M. vaccae has demonstrated that the G+C (guanine plus cytosine) contents of the DNAs are 56, 61, and 65%, respectively, and that the genome sizes are 2.2×10^9, 3.1×10^9, and 3.1×10^9 dalt respectively. Because of the significant differences in both G+C content and genome size among M. leprae, M. "lufu" and M. vaccae DNAs, these species are not related, although hybridization experiments under non-stringent conditions, using two separate cloned M. leprae DNA inserts as probes, indicate that there are some conserved sequences among the DNAs. The G+C content of Dasypus novemcinctus (armadillo, the animal of choice for cultivating M. leprae) DNA was determined to be 36%. Genomic libraries potentially representing greater than 99.99% of each genome were prepared by cloning into the cosmid vector, pHC79, in Escherichia coli K-12. A genomic library representing approximately 95% of the genome of M. vaccae was prepared in pBR322. M. leprae DNA was subcloned from the pHC79::M. leprae library into an expression vector, pYA626. This vector is a 3.8-kilobase derivative of pBR322 in which the promoter region of the asd (aspartate semialdehyde dehydrogenase) gene from Streptococcus mutans has been inserted in place of the EcoRI to PstI fragment of pBR322. Several (44% of those tested) pYA626::M. leprae recombinants and one pBR322::M. vaccae recombinant synthesized new polypeptides in minicells of E. coli, indicating that mycobacterial

DNA can be expressed in E. coli K-12, although expression is probably dependent upon use of non-mycobacterial promoters recognized by the E. coli transcription-translation apparatus.

INTRODUCTION

Although Mycobacterium leprae was one of the first microorganisms to be implicated as the causative agent of a disease (leprosy), inability to grow this organism in the laboratory has greatly hampered studies on its genetics, physiology and mechanism(s) of pathogenicity. In 1971, Kirchheimer and Storrs (21,38) reported the successful experimental infection of armadillos (Dasypus novemcinctus, Linn.) and since that time, experimentally infected armadillos have been a source of M. leprae for investigators throughout the world. Because available supplies of M. leprae are limited, investigators have attempted to identify other mycobacterial species that share antigens or antigenic determinants with M. leprae (e.g., M. vaccae, M. bovis BCG, M. lepraemurium) and thus might be used for development of a vaccine against leprosy (16,37). Other investigators have used other mycobacterial species (e.g., Mycobacterium "lufu") with drug sensitivity profiles similar to M. leprae as model systems for determining the mode of action of diaminodiphenylsulfone (dapsone), the drug of choice for treating leprosy, and for testing the efficacy of new drugs in the treatment of this disease (30,34).

Recombinant DNA technology offers an obvious advantage for studying the genetics and physiology of M. leprae, provided that M. leprae genes are expressed in the host bacterial strain. In preparation for cloning M. leprae DNA, the mole per cent G+C (guanine + cytosine content) and genome sizes of M. leprae, M. vaccae, and M. "lufu" DNAs were determined. In addition, it was important to determine the G+C content of armadillo DNA in order to ensure separation of M.

leprae DNA from any residual armadillo DNA prior to cloning. Moreover, the percent G+C of this DNA has not been reported.

Recombinant banks or libraries of M. vaccae, M. "lufu" and M. leprae DNAs were prepared, using the cosmid cloning vector, pHC79 (18). In In this paper, we report the initial molecular analysis of these three mycobacterial DNAs, the construction of the cosmid libraries and evidence for expression of mycobacterial DNA in Escherichia coli K-12.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. E. coli K-12 strains χ925, χ2001 and χ2819 were used as host strains for preparing the recombinant cosmid and plasmid libraries. All other bacterial strains were used as sources of DNA.

Armadillo tissue. An uninfected armadillo was obtained from the Singleton Trapping Company, Riverview, Fla., since armadillos with naturally-occurring leprosy-like infections have not been observed in Florida. This animal was sacrificed, its liver was aseptically removed and used as a source of uninfected armadillo DNA.

Media and Reagents. The cultivable bateria were grown in the following media: E. coli and Pseudomonas aeruginosa in L broth (24) or minimal salts broth or agar supplemented with amino acids and glucose (9); M. vaccae in TB broth (Difco, Detroit, Mich.) and M. "lufu" in modified Dubos-Davis broth (Difco); the last two media were supplemented with 0.25% (wt/vol) bovine serum albumin fraction V (Difco).

Reagents for separation of M. leprae cells from armadillo tissue were those recommended by Draper (14). Buffer A contained 0.15 M NaCl, 0.015 M HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) and 1 mM $MgSO_4$ and buffer B contained 0.15 M NaCl, 0.2 M Tris buffer and 1 mM $MgSO_4$. Both buffers were adjusted to pH 7.2. Buffered saline with Tween (BST) contained 0.15 M NaCl, 2.2 mM KH_2PO_4, and 4.2 mM Na_2HPO_4 (pH 7.0). Each of these three buffers

contained 1 mM benzamidine as an inhibitor of armadillo cellular proteases (at the suggestion of Dr. T. Buchanan) and 0.2% (wt/vol) Tween 80. Percoll (Pharmacia Fine Chemicals, Piscatoway, N.J.), a colloidal suspension of silica particles coated with polyvinylpyrrolidone, was used to form density gradients. Polyethylene glycol (PEG)-palmitate-dextran was a phase mixture in which 7.0 g Dextran T500, 4.9 g PEG 6000 and 0.1 g PEG-palmitate (a generous gift of Dr. P. Brennan) were added to 63 ml sterile 0.01 M potassium phosphate buffer (pH 6.9)-0.01 M NaCl and allowed to dissolve overnight.

Reagents for DNA isolation were described by Marmur (28). Saline-EDTA was 0.15 M NaCl-0.01 M EDTA (pH 8.0). Reagents used for deproteinization were 5 M $NaClO_4$ and Sevag solution (24 parts chloroform to 1 part isoamyl alcohol). Standard saline-citrate (SSC) was 0.15 M NaCl-0.015 M sodium citrate (pH 7).

All restriction endonucleases and T4 ligase were obtained from Bethesda Research Laboratories, Bethesda, Md., or New England Biolabs, Boston, Mass. All other enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions of proteinase K (protease XI; 10 mg/ml) and ribonuclease A (2 mg/ml) were divided into 1 ml aliquots and stored frozen at -20°C. Prior to freezing, RNase stock solution was boiled for 10 min and then slowly cooled to inactivate DNases.

Separation of M. leprae cells from armadillo liver tissue. The method for quantitative recovery of M. leprae cells from infected armadillo liver was adapted from a method developed by Draper (14) and Shepard et al. (36).

Tissue homogenization was accomplished by cutting a 15-g sample of infected liver into pieces of approximately 1 cm^3 which were placed into a sterile 200-ml stainless steel Omnimixer cup (Sorvall Instruments, Norwalk, Conn.) with 60 ml of ice cold Buffer A. While the cup was immersed in ice, the tissue was homogenized for a total of 5 min (1 min of homogenization alternated with 1 min of rest) at top speed and then allowed to rest for 20 min. This homogenate was then transferred to sterile 30-ml Oak Ridge polycarbonate centrifuge tubes and spun at 12,000 rpm in a Sorvall SS34 rotor in an RC-5B centrifuge for 10 min at $4^{o}C$. (All subsequent sedimentations throughout the separation procedure were done in 30-ml polycarbonate Oak Ridge tubes in a Sorvall SS34 rotor.) As much of the the supernatant fractions as possible was carefully removed (and discarded) without disturbing the pellets. The pellets were mixed with residual supernatant fluid with the piston of a Pyrex tissue grinder(13 by 100 mm) and were then suspended in 10 ml of Buffer A (total) by pipetting up and down with a 10 ml pipette until clumps were suspended. All suspended cells were transferred to the Omnimixer cup and the centrifuge tubes were rinsed with Buffer A until a final volume of 60 ml was obtained. All washes were added to the Omnimixer cup. This mixture was homogenized for an additional 3 min, allowed to rest, and centrifuged as described above. As much of the supernatant fractions as possible was again carefully removed (and discarded) and the pellets were completely suspended in the residual supernatant fluid plus 10 ml of fresh Buffer A as described above. This mixture was passed through a sterilized wire mesh tea strainer (diameter, 3 in.) (to remove tissue strands) into a

sterile 250 ml beaker. The contents of the beaker were then transferred to a sterile 250-ml screw-capped flask. Sufficient Buffer A to make a final volume of 60 ml was used to thoroughly wash the centrifuge tubes, strainer and beaker; all washes were added to the flask containing the homogenized mixture.

Enzymatic digestion of armadillo collagen and DNA was accomplished by adding 0.2 ml of 1 M $CaCl_2$ and 6 mg of collagenase to the flask containing the homogenized mixture. The flask was incubated at $30^{o}C$ with gentle aeration for 1 h. During a second hour of incubation, 0.6 mg of DNase was added to the mixture to digest armadllo DNA. The mixture was then transferred to sterile tubes and centrifuged at 12,000 rpm for 10 min. The supernatant fractions were carefully removed, and each pellet was suspended by vortexing in 5 ml of Buffer B. The mixture can be stored overnight at $4^{o}C$ at this point. In the morning, 10 ml of Buffer B was added to each tube and the tubes centrifuged at 12,000 rpm for 10 min. The supernatant fractions were removed, the pellets were mixed with the tissue grinder piston, suspended in a total of 5 ml of BST and the mixture was divided equally among six centrifuge tubes.

Density separation of M. leprae cells from armadillo tissue residue was accomplished by Percoll gradient separation with two different densities of Percoll. BST was added to each tube to bring the total volume to 9.6 ml per tube. Percoll (10.4 ml) was added to each tube over a period of approximately 2 min per tube (to avoid osmotic shock to the M. leprae cells) to give a final concentration of 52%. The six tubes were centrifuged at 15,000 rpm for 15 min at

4°C. The gradient formed has a number of bands (all measured in distance below the meniscus): (i) a white milky band that extends from the meniscus to approximately 3 mm, (ii) a brown liver cell band from 3 to 10 mm, (iii) an area of light brown turbidity from 10 to 14 mm, (iv) a relatively clear area from 14 to 27 mm, (v) a band containing M. leprae cells (which starts out slightly turbid, then increases to a heavier band) from 27 to 46 mm and (vi) a brown band from 46 to 49 mm. The top three bands and some of the clear portion were carefully removed from each tube with a Pasteur pipette; BST was slowly added to each tube until the total volume per tube was 15 ml. The tubes were mixed gently by inversion and were then centrifuged at 12,000 rpm for 10 min at 4°C. As much supernatant fraction as possible was removed from each tube, the pellets were gently suspended in BST, and the mixture was divided evenly into four centrifuge tubes. The volume of BST in each tube was adjusted to 15 ml and the tubes were centrifuged at 12,000 rpm for 10 min. At this point, the supernatant fractions were completely removed and each pellet was suspended in BST. The density of the next Percoll gradient was dependent upon the extent of separation between liver cells and M. leprae cells achieved in the first gradient. If band iv was turbid, the four pellets were each suspended in 9.6 ml BST and a second 52% Percoll gradient was run, as described above. When band iv was clear and bands v and vi overlapped, separation was on an 80% Percoll gradient. The pellets recovered from the 52% gradient were each suspended in 4 ml of BST. To each tube, 16 ml of Percoll was slowly added (to give a final concentration of 80% Percoll) and the four

tubes were centrifuged at 15,000 rpm for 15 min. The gradient formed this time had the following bands (all measured in distance below the meniscus): (i) a brown layer at the meniscus; (ii) a white or beige layer of M. leprae cells from 2 to 5 mm below the meniscus (heavy band) plus a more diffuse band from 5 to 10 mm, (iii) a clear area from 10 to 35 mm , (iv) a diffuse brown band (containing liver tissue) from 35 to 48 mm, and (v) a pellet of brown and white material. The top two bands (containing M. leprae cells) were removed with a Pasteur pipette, diluted with BST, mixed by inversion and centrifuged as they were after the first Percoll gradient. The pellets were washed with BST and centrifuged as described above; after the centrifugation, the pellets were suspended and combined in a total of 20 ml of BST.

The final purification of the M. leprae cells was achieved by phase separation. The cell suspension in BST was added to 80 ml of PEG-palmitate-dextran in a 250-ml separatory funnel, which was then inverted 100 times. The contents of the funnel were allowed to separate for approximately 30 min. More M. leprae cells can be recovered if they are collected when the liver band (which is in the lower half of the separatory funnel) is approximately 2 cm wide than if the liver band forms more tightly. The PEG-palmitate layer (cloudy white) was removed from the top of the funnel with a Pasteur pipette, added to two sterile centrifuge tubes and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant fraction was removed and added to the dextran in the separatory funnel, and the phase separation repeated two to four times (as long as good pellets of M. leprae

cells were recovered after centrifugation of the PEG-palmitate layer). After the final phase separation, all cell pellets were combined and suspended in a total of 15 ml of BST-1.5 ml of dimethyl sulfoxide in a polycarbonate centrifuge tube. The cells were frozen slowly at -70°C until used for DNA isolation. A 20µl sample of the cells was removed before the addition of dimethyl sulfoxide for determining the titer of acid-fast bacilli.

The liver bands from the two Percoll gradients were also collected and washed with BST until good pellets were formed. All pellets were combined and suspended in a total of 15 ml of BST. A sample was removed for acid-fast staining to determine whether or not a significant number of M. leprae cells remained associated with the liver residues. If a significant titer of M. leprae cells was still present, the suspension was put through the PEG-palmitate/dextran phase separation to recover the M. leprae cells.

Isolation of DNA. E. coli, P. aeruginosa, and armadillo DNA were isolated by the Marmur procedure (28). To extract DNA from M. leprae, frozen cells from three separation procedures (1×10^{11} to 2×10^{11} cells) were thawed, centrifuged at 10,000 rpm for 10 min at 4°C, washed with saline-EDTA, and sedimented by centrifugation with complete removal of the supernatant fluid. The centrifuge tube was placed on dry ice and the frozen pellet of cells was removed from the tube. M. vaccae and M. "lufu" cells were frozen at -20°C after harvesting from their respective growth media. DNA was isolated from the mycobacteria as follows: frozen cells were placed in a mortar which contained dry ice and glass beads (0.1 g of 20µm-diameter

beads; 3M Co., Minneapolis, Minn). The cells were triturated for approximately 15 min (until the cells were ground to a fine powder-like consistency and the dry ice had sublimed) and were then transferred to a sterile screw-capped 125-ml flask. The mortar was allowed to warm to room temperature and then 10 ml of saline-EDTA was used to wash any residual cells out of the mortar into the screw-capped flask. Egg white lysozyme was added to a final concentration of 200μg/ml and the flask was incubated at 37°C with moderate aeration (e.g., 100-150 rpm on a platform shaker; Lab-Line Instruments Inc., Melrose Park, Ill.) for 1 h. Proteinase K was added to the flask to a final concentration of 250μg/ml and the flask was incubated at 60 to 65°C for 15 min with occasional gentle swirling. Sodium dodecyl sulfate (SDS) was added to a final concentration of approximately 3.5% (vol/vol) and the flask was incubated at 60 to 65°C for an additional 15 min with occasional gentle swirling. The flask was quickly cooled to room temperature by running cold water over it, and then $NaClO_4$ was added to a final concentration of 1 M. The flask was shaken gently (e.g., 75 rpm on a Lab-Line platform shaker) for 5 min at room temperature or 37°C. A volume of Sevag solution equal to the contents of the flask was added and gentle shaking continued for 15 min (at either room temperature or 37°C). The contents of the flask were transferred to chloroform-resistant 50-ml Oak Ridge centrifuge tubes and centrifuged at 10,000 rpm in a Sorvall SS34 rotor for 15 min at 4°C. The top aqueous layer was removed from each tube and placed in silicanized 30-ml Corex centrifuge tubes. The nucleic acids were recovered from these tubes

by ethanol precipitation, suspended in 4.5 ml of 0.015 M NaCl-0.0015 M sodium citrate (when the nucleic acids were in solution, the salt concentration was adjusted to 0.15 M NaCl-0.015 M sodium citrate), and transferred to a 50-ml screw-capped flask. RNase was added to a final concentration of 50μg/ml and the flask was incubated at 37°C with gentle shaking for 1 h. Proteinase K was added to a final concentration of 100μg/ml and the flask was incubated at 60 to 65°C for 15 min with occasional gentle swirling. The flask was cooled to room temperature and 0.98 g cesium chloride (Kawecki Berylco Industries, Inc., Reading, Pa.) per ml of DNA solution was added. The solution was transferred to Beckman VTi65 Quik-seal tubes (Beckman Instruments, Inc., Palo Alto, Calif.), and 2.5 mg ethidium bromide was added to each tube. The tubes were then centrifuged in a Beckman VTi65 rotor in an ultracentrifuge for 20 h at 45,000 rpm at 14°C. After centrifugation, the DNA bands were visually located using a short wavelength UV light and were recovered from the gradient. The bands were combined and run on a second cesium chloride-ethidium bromide gradient as described above. After collecting the bands from the second gradient, the ethidium bromide was removed with NaCl-saturated isopropanol (27) and the DNA was then dialyzed at 4°C against 10 mM Tris buffer (pH 8): three changes of 250 to 300 ml buffer over an 18-h period. The DNA was removed from the dialysis tubing and placed in a screw-capped tube. EDTA (pH 8) was added to a final concentration of 1 mM, and the DNA was stored at 4°C.

Determination of G+C content. The G+C content of each DNA was determined from the midpoint of the melting curve measured optically

at 260 nm (Tm) and from the isopycnic bouyant density of the DNA (32). The Tms of the DNAs were determined in 0.015 M NaCl-0.0015 M sodium citrate by the method of Marmur and Doty (29) using a Gilford Model 250 spectrophotometer equipped with a Model 2257 Thermoprogrammer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). E. coli K-12 χ289 DNA was used as a reference standard. The G+C content of E. coli K-12 x289 was calculated from the equation % G+C = 100 [(Tm/50.2) -0.990] and the G+C contents of the other DNAs were calculated from the equation % G+C of unknown = 100 [G+C of E. coli + 0.0199 (Tm of unknown -Tm of E. coli)] (26).

The bouyant densities were determined by isopycnic ultracentrifugation in a Beckman Model E ultracentrifuge, with M. luteus DNA (a gift of J. Lebowitz) as a reference standard. These densities were determined by Cheryl Goguen, Department of Microbiology, University of Alabama in Birmingham. The G+C contents of the DNA samples were determined from their bouyant densities by the equation % G+C = 100 [(ρ - 1.660)/0.098] (32).

Determination of genome sizes. Genome sizes of the mycobacterial DNAs were determined from the rates of reassociation of denatured DNA (C_0t analysis) as measured optically in the Gilford spectrophotometer (5,6,15). Each DNA sample was sheared by sonication of 10-s bursts alternating with 10-s rests for a total of 1 min. Sonication was done with the small probe of a Braun Sonic Model 1510 (B. Braun Melsungen AG) at highest power (100 watts). The DNA samples were suspended in 1.2 M NaCl-0.12 M sodium citrate and the tubes containing the DNA were submerged in ice throughout the sonication. The DNA was diluted to

40μg/ml and formamide was added to a final solvent concentration of 25% (vol/vol) in 0.9 M NaCl-0.09 M sodium citrate (5). Denaturation and reassociation of the DNA samples were performed as described by Bradley (5). Absorbance of the DNA samples at 270 nm was monitored until the denatured DNA was at least 65% reassociated. E. coli χ289 DNA was the reference standard for the reassociation experiments. Genome sizes were determined from the equation - $C_0t_{1/2}$ of E. coli/$C_0t_{1/2}$ of unknown = (2.5×10^9)/molecular weight of unk DNA (15). A factor derived by Seidler and Mandel (33) was used to correct for the differences in the G+C content between the mycobacterial DNAs and E. coli DNA.

The DNA from the total pHC79::M. leprae recombinant library was sheared denatured, and reassociated in the same way as the chromosomal DNAs in order to estimate the fraction of the M. leprae genome that is represented in the recombinant library. Sheared DNA from pHC79 was simultaneously denatured and reassociated as a control.

Construction of genomic libraries. To prepare DNA for cosmid cloning, mycobacterial and pHC79 DNAs were digested with restriction endonucleases according to published procedures (27). M. "lufu" DNA was completely digested (in separate reactions) with HindIII and EcoRI; M. leprae and M. vaccae DNAs were partially digested with PstI. The vector DNA was completely digested with the appropriate restriction enzyme and then was treated with alkaline phosphatase to inhibit self-ligation (27).

The chromosomal DNA fragments were fractionated on linear sucrose gradients. Sucrose solutions of 5% and 25% (wt/vol) were prepared in

10 mM Tris, 1 mM EDTA, pH 8.0 (TE) buffer and used to form 4.0 ml linear gradients in Beckman SW56 nitrocellulose centrifuge tubes. The digested DNAs (at concentrations of 20μg in 200μl) were layered on top of separate gradients. The tubes were centrifuged at 34,000 rpm in a Beckman SW56 rotor for 10.5 h at 10°C. The gradients were fractionated by puncturing the bottom of the tubes with an 18-gauge needle and collecting 2 to 3 drop fractions. The DNA was recovered by ethanol precipitation, using 20μg of yeast carrier RNA per fraction. Since the RNA did not appear to inhibit ligation, no effort was made to eliminate it. The sizes of the digested DNA fragments were estimated from 0.4% agarose (Type 1; Sigma) gels run at 2V/cm. Molecular weight standards included in the gels were DNA from bacteriophages T4, T5, T7, and λ and λ DNA digested with the restriction endonuclease SalI.

For M. leprae DNA, two types of ligations were done: (i) the 15-kilobase (kb) fragments plus the 30-kb fragments were mixed with pHC79 and (ii) the 20-kb fragments were mixed with pHC79. For M. vaccae DNA, only the 20-kb fragments were mixed with pHC79. M. "lufu" HindIII-restricted or EcoRI-restricted DNA fragments were not separated by size on sucrose gradients; an aliquot of each DNA digestion mixture was ligated to pHC79 DNA that had been digested with the same restriction endonuclease. In all ligation mixtures the ratio of chromosomal DNA to vector DNA was 2 to 1 (mole:mole) and approximately 0.5 U of bacteriophage T4 ligase was added to each 20μl-reaction mixture (containing a total of 2.5 to 10μg of DNA). All ligation reactions were done at 16°C for 16 h.

The recombinant cosmids were packaged in vitro (8) and were used to transduce χ2001 or χ2819. After the in vitro-packaged cosmids had adsorbed to the cells, the cultures were incubated at 30°C for 30 min and the transductants were selected for ability to grow on L agar containing (i) 25μg Ap per ml (final concentration) when HindIII or EcoRI-digested M."lufu" DNAs were used or (ii) 5.0μg tetracycline per ml (final concentration) when PstI-digested M. leprae or M. vaccae DNAs were involved in the transduction. The Ap^r colonies obtained following transduction with the HindIII-digested DNA were tested for sensitivity to 12.5μg tetracycline per ml and the Tc^r colonies obtained following transduction with the PstI-digested DNA were tested for sensitivity to 12.5μg Ap per ml. Although a portion of the Ap^rTc^r colonies obtained from the HindIII-digested reaction mixture could have had M. "lufu" DNA inserts, analysis of the recombinant molecules was confined to those which were Ap^rTc^s. Colonies that were Ap^rTc^s from the HindIII-digested mixture were grown in L broth containing 12.5μg ampicillin per ml and colonies that were Tc^rAp^s from the PstI-digested mixtures were grown in L broth containing 5.0μg tetracycline per ml; cosmid DNA was extracted from both types of recombinants by the method of Birnboim and Doly (3) or Birnboim (2). Both undigested and endonuclease-digested DNA samples were analyzed by electrophoresis on 0.7% agarose gels.

Recombinant cosmid libraries prepared in χ2001 were stored frozen at -70°C in 1% peptone broth plus 5% glycerol as plasmid-containing cells. When the recombinant cosmid libraries were introduced into χ2819, the transductants were grown to high titers by taking

advantage of the presence of the λ prophage in this host strain. The mutations in the prophage (i) reduce the prophage's ability to integrate into and to be excised from the chromosome of the host cell (b2), (ii) preclude recombination between the prophage and the recombinant cosmid (red3), (iii) preclude lysis in a suppressor-free host cell (S7), and (iv) allow for thermal induction of phage protein synthesis (cI857). Thus, when a transductant culture is thermally induced, the only DNA available for packaging is that of the recombinant cosmids. Since χ2819 is tyrT$^+$, cell lysis by the prophage is prevented, so the cells become elongated, filled with in vivo-packaged recombinant cosmids until they are lysed externally (W.R. Jacobs, J.E. Clark-Curtiss, L.R. Ritchie, and R. Curtiss III, Abst. Annu. Meet. Am. Soc. Microbiol. 1983, H147, p. 130). Lysates containing 10^{10} packaged recombinant cosmids per ml were prepared by concentrating the transduced χ2819 cells by centrifugation at 4000 × g for 10 min at 4°C, gently suspending the pellets in 1/10 to 1/50 the original volume and lysing the cells by adding 0.01 volume chloroform and vigorously shaking the culture at 37°C for 10 min. In some cases, these lysates were further concentrated to titers of 10^{11} Tc or Ap resistance-conferring transducing phage particles per ml by centrifugation through CsCl. Purified lysates were stored at 4°C.

To enhance the possibility of expression of cloned M. leprae DNA in E. coli, M. leprae DNA was subcloned from the pHC79::M. leprae libraries into an expression vector, pYA626 (a 3.8-kb plasmid containing the promoter region from the Streptococcus mutans asd gene

(13,19) cloned in between the PstI and EcoRI sites of pBR322, constructed by Guy Cardineau and Roy Curtiss). Recombinant cosmids containing M. leprae DNA were isolated as plasmid DNA (2,3) from a pool of Tc^r Ap^s colonies and were completely digested with PstI. These fragments were mixed with PstI-digested pYA626 DNA and T4 ligase. The resulting pYA626::M. leprae recombinant plasmids were transformed (27) into E. coli K-12 χ925 and transformants were selected on L agar containing 5.0μg tetracycline per ml. These transformants were again tested for sensitivity to 12.5μg ampicillin per ml and the plasmid DNA of the Tc^r Ap^s transformants was analyzed by agarose gel electrophoresis as described above.

A recombinant library of M. vaccae chromosomal DNA was prepared in the plasmid cloning vector, pBR322 (4). M. vaccae and pBR322 DNAs were digested to completion with the restriction endonuclease BamHI, ligated as described above and the recombinant molecules were transformed (12) into E. coli χ1849. Transformant colonies were selected for growth on L agar containing 25μg ampicillin per ml; these were tested for sensitivity to tetracycline as described above. Recombinant DNA was extracted from Ap^r Tc^s transformants and analyzed by agarose gel electrophoresis as described above.

Hybridization of probe DNA to chromosomal DNA. Two different pYA626::M. leprae recombinant molecules (pYA1026 and pYA1031) were completely digested with PstI and the fragments were separated by electrophoresis in 0.7% agarose (Type 1, Sigma). The M. leprae insert DNA fragments from each probe were recovered by removing the appropriate slices from the gel and electroeluting the DNA as

described by Maniatis et al. (27). The DNA fragments were then concentrated by passage through a NACS Prepac (Bethesda Research Laboratories), ethanol-precipitated and nick-translated, using [α-^{32}P]-dATP (27).

Chromosomal DNAs from E. coli, armadillo, M. leprae, M. "lufu", and M. vaccae were each completely digested with PstI and were separated by electrophoresis on a 0.7% agarose gel (2 V/cm). The digested DNAs were transferred from the agarose gel to a filter of GeneScreenPlus (New England Nuclear, Boston, Mass.) by capillary action, using 10×SSC as the transfer buffer, as described by Maniatis et al. (27).

The labelled probe DNAs were denatured by boiling for 10 min, followed by immersion in ice. The chromosomal DNAs fixed to the GeneScreenPlus filters were prehybridized for 20 h at 65°C, sealed in plastic bags with 10 ml of a solution containing 1% SDS, 1 M NaCl and 10% dextran sulfate per filter. The denatured radioactive probes were added at a concentration of 10ng/ml (final concentration in the reaction mixutre) together with denatured salmon sperm DNA (final concentration, 100µg/ml) to separate filters. The plastic bags were resealed and incubated at 65°C with continuous agitation for 24 h. After washing the filters twice with 2×SSC at room temperature for 5 min each, twice with 2×SSC plus 1% SDS at 65°C for 30 min each, and twice with 0.1×SSC at room temperature for 30 min each, the filters were air-dried briefly and were then autoradiographed using Kodak XAR-2 film (Eastman-Kodak, Rochester, N.Y.) for 18 h.

Complementation analysis of recombinant molecules. Recombinant molecules were tested to determine whether or not the cloned DNA contained genes that could complement any of the genetic defects present in the host E. coli strains. This was done by plating 10^7 to 10^8 transformed or transduced E. coli cells on minimal salts agar either lacking one required growth supplement or containing one non-metabolizable carbon source. All media contained either 12.5μg Ap per ml or 5.0μg Tc per ml.

Minicell analysis of recombinant plasmids. E. coli K-12 χ925 cells containing the pYA626::M. leprae recombinants or χ1849 cells containing pBR322::M. vaccae recombinants were grown to late log phase in minimal salts broth supplemented with 0.5% glucose, thiamine and all amino acids except methionine (minimal salts broth growth medium). The minicells (1) from the cultures were separated from the parental cells by differential centrifugation followed by sedimentation through one or two sterile linear 5 to 20% sucrose in buffered saline plus gelatin gradients (7,31). The final minicell preparations had less than one contaminating parental cell per 10^7 minicells after two sucrose gradients. The minicells were suspended in 1 ml fresh minimal salts broth growth medium in a sterile Eppendorf tube, incubated for 10 min at 37^0C and were then labeled with 10μCi of [^{35}S]-methionine (1000 Ci/mmol) for 2 min. The reaction was stopped by immersing the minicell preparation in ice water for 30 min. The minicells were sedimented by a 2 min centrifugation in a Beckman Microfuge and the minicell pellet was suspended in 50μl of Laemmli buffer (23). This suspension was boiled at 100^0C for 2 min to lyse

the minicells. A 5µl sample was removed from each suspension, spotted on a Whatman 3 MM filter and assayed for incorporation of the [^{35}S]-methionine into hot trichloroacetic acid-insoluble material. The remaining 45µl of the lysed minicell preparations were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (23).

RESULTS

Separation of M. leprae cells from armadillo liver tissue. Using the method for purifying M. leprae cells from armadillo liver tissue described in this paper, 92 to 100% of the cells in the tissue were recovered, based on microscopic counts of the acid-fast bacteria in the tissue versus those in the purified preparation.

Isolation of M. leprae DNA. Approximately 1 x 10^{11} to 2 x10^{11} purified M. leprae cells were used for the DNA isolation procedure, from which we were able to recover 60% of the DNA present in those cells, assuming that each cell had only a single chromosome of 2.2 x 10^{9}. Using the viability stain developed by Kvach and Veras (22), we determined that 60 to 65% of the acid-fast bacteria recovered from the armadillo tissue were viable (i.e., had intact cytoplasmic membranes). Thus, the amount of DNA recovered from these cell preparations may be the maximal amount recoverable, since nucleases may have degraded the DNA in the non-viable cells. Analysis of the M. leprae DNA by electrophoresis on 0.3% agarose gels, using bacteriophage T4 (178-kb), T5 (110-kb) and lambda (48.6-kb) DNAs as molecular weight standards, indicated that the M. leprae DNA fragments are 100 to 200 kb in length (data not shown).

Determination of G+C content and genome sizes of mycobacterial DNA. The G+C content of the DNAs, determined by thermal denaturation and by isopycnic bouyant density centrifugation, are given in Table 2. Thermal denaturations were conducted at least two and as many as six times, whereas the bouyant densities were usually determined only

once. E. coli K-12 χ289 DNA served as the reference standard for the Tm determinations and Micrococcus luteus DNA as the reference standard for the bouyant density determinations. Each of these DNAs was analyzed by the alternative method as well. P. aeruginosa DNA was included as an additional control, since the published values for its G+C content are similar to those reported for mycobacterial species (39), although the two genera are unrelated.

In most cases, there is a very good agreement between the values determined by the two methods. Armadillo DNA has a G+C content slightly lower than those reported for other mammals (39 to 44% [35]), which is significantly different from that of M. leprae. M. vaccae DNA has a G+C content similar to the reported values for other mycobacterial DNAs, which fall into two groups: one with G+C contents of 64 to 66.4% and the other with G+C contents of 67 to 70% (39). M. leprae DNA, in contrast, has a G+C content significantly different from other mycobacteria, as has been reported by Imaeda et al. (19). M. "lufu" DNA also has a G+C content that is lower than other mycobacterial DNAs.

The molecular weights of M. "lufu" and M. vaccae genomes are similar to those of a variety of mycobacterial species reported by Bradley (5). The M. leprae genome is slightly smaller than other mycobacteria.

The molecular weight of the pHC79::M. leprae recombinant library estimated from the $C_0t_{1/2}$ value was 1.9×10^9 using t correction factor for the difference in G+C content between M. leprae and E. coli chromosomal DNAs. The pHC79 DNA alone reassociated very

quickly: this DNA was more than 50% reassociated by the time the temperature within the cuvette chamber had dropped to and stabilized at the reassociation temperature for the chromosomal DNA (2.5 to 3 min).

Preparation of genomic libraries. Concurrently with the molecular analysis, we prepared recombinant libraries, initially with M. vaccae and M. "lufu" DNAs, since these DNAs were more plentiful, and then with M. leprae DNA. Recombinant cosmid libraries prepared from these DNAs each consist of apprpximately 1000 recombinant molecules with inserts of mycobacterial DNA of 40 to 45 kb. Thus, each library theoretically represents greater than 99.99% of the respective mycobacterial genome. In addition, a pBR322::M. vaccae library was prepared which consists of 1500 recombinant molecules with inserts of 0.8 to 19.2 kb of M. vaccae DNA, and which potentially represents 95% of the M. vaccae genome.

Each library was screened for complementation of the following genetic defects in the host E. coli K-12 strains: proA, trpE, thyA, asd, araC, and lacZ. No complementation of any of these defects by mycobacterial DNA has been detected.

Expression of mycobacterial DNA in E. coli. The M. leprae DNA was therefore subcloned into the PstI-site of the plasmid expression vector pYA626. This vector contains the promoter region from the S. mutans asd gene; when the entire S. mutans gene is present in the plasmid (pYA575), the asd gene product comprises 7% of the total protein produced by whole E. coli cells (13). Moreover, the asd promoter apparently has a greater affinity for E. coli RNA polymerase

than does the β-lactamase (bla) promoter from the pBR322 portion of the plasmid in that the synthesis of β-lactamase is almost totally shut off in minicells (which have a limited amount of RNA polymerase) harboring pYA575 (13,20). The asd promoter is therefore an extremely strong promoter and we anticipated that subcloning M. leprae DNA into such an expression vector would enhance expression of M. leprae genes in E. coli. The recombinant molecules were transformed into E. coli K-12 χ925 and tetracycline-resistant transformants were recovered at a frequency of 5×10^3 per μg of DNA. Six hundred transformants were screened for sensitivity to ampicillin: approximately 50% were sensitive. Since the subcloned DNA was from the PstI-digested pHC79::M. leprae library, several types of recombinant molecules could be formed: (i) re-ligated pHC79, (ii) pHC79::M. leprae DNA, (iii) pHC79::pYA626, and (iv) pYA626::M. leprae DNA. The transformants that were Tc^r Ap^r were cells that inherited the re-ligated pHC79 molecules. Of the recombinant molecules analyzed, 80% contained inserts of sizes different than 6 kb (the size of pHC79). Figures 1A and 1B are photographs of 0.7% agarose gels on which plasmid DNAs from 11 of these transformants have been analyzed: Fig. 1A depicts the intact recombinant molecules and Fig. 1B depicts a gel on which PstI-digested DNA from the same sources was analyzed. The inserted DNA present in most of these recombinant molecules ranges in size from approximately 1.4 to 10.5 kb except pYA1025, in which two bands of approximately 22 and 25 kb were faintly visible on the original photograph of the gel. It is evident from Fig. 1B that none of the 11 recombinant cosmids contained an insert of the size of

pHC79, with the possible exception of pYA1035 (Lane 15) which gave a doublet band with fragments of approximately 6.7 and 6.9 kb in size.

Hybridization of the M. leprae 2.8 kb insert DNA fragment from pYA1031 (labeled with ^{32}P) to chromosomal DNA is shown in Figure 2. Figure 2A is a photograph of a 0.7% agarose gel on which PstI-digested chromosomal DNAs from E. coli, armadillo, M. leprae, M. "lufu", and M. vaccae were separated. Figure 2B depicts the autoradiograph showing that this insert DNA hybridized only with M. leprae chromosomal DNA. The larger (2.1 kb) fragment of pYA1026 and the 2.5 kb insert of pYA1036 each hybridize slightly to M. "lufu" chromosomal DNA and less well to M. vaccae chromosomal DNA, indicating that there are some conserved sequences present in the genomes of these mycobacterial DNAs (data not shown).

Minicells were then isolated from nine of the eleven transformants and the polypeptides produced by the recombinant molecules were labeled with [^{35}S]methionine and analyzed by SDS-PAGE. Figure 3A and 3B are autoradiographs of the gels, which show that at least four of the recombinant molecules (pYA1025, pYA1026, pYA1028, and pYA1031) specified polypeptides in addition to the 33 kilodalton (kDa) product of the tetracycline resistance gene of the vector, which is shown in Lane 8 of each gel. Two of the recombinant molecules specified multiple polypeptides: pYA1025 specified polypeptides of 40 and 52 kDa and pYA1028 specified polypeptides of 20, 37 and 40 kdal.

Minicells were also isolated from E. coli χ1849 isolates containing two of the pBR322::M. vaccae molecules (pYA1001 and pYA1002). The polypeptide specified by these recombinant molecules

were labeled and analyzed by SDS-PAGE. Figure 4 is an autoradiograph of this gel which demonstrates that pYA1001 specifies a new polypeptide of approximately 50 kDa.

DISCUSSION

The base composition and genome size determinations on M. vaccae and M. "lufu" DNAs were initiated because both of these organisms are cultivable (and thus provide easily accessible DNA in adequate amounts) and because each has been reported to show similarities to M. leprae (16,30,34,37). One or both of these organisms could then be used to optimize procedures for preparing genomic libraries of mycobacterial DNA and for obtaining expression of mycobacterial DNA in E. coli K-12.

As is evident from the data in Table 2, the M. leprae genome is significantly different from the genomes of M. vaccae and M. "lufu", as well as other mycobacteria that have been studied, both in base composition and in molecular weight of the genome. Bradley (5) reported genome sizes of 2.8×10^9 to 4.5×10^9 daltons for ten different mycobacterial species; only Mycobacterium tuberculosis H37Ra, with a genome of 2.5×10^9 daltons, was similar to that of M. leprae determined in this study. (However, M. tuberculosis has a G+C content of 65% [5].)

M. vaccae DNA is similar to other mycobacterial DNAs in G+C content and molecular weight of its genome. The size of the M. "lufu" genome is similar to those of other mycobacteria, although the G+C content of its DNA is lower. Little homology (less than 15%) has been observed in filter hybridization experiments (under non-stringent conditions) between total chromosomal DNA from M. leprae and chromosomal DNA from either M. "lufu" or M. vaccae (C. Grosskinsky

and B. Bloom, personal communication). Thus, we conclude that M. vaccae and M. "lufu" are not closely related to M. leprae, although hybridization experiments (under non-stringent conditions) with two independent cloned M. leprae inserts demonstrate that there are specific sequences that have been somewhat conserved among the three species.

The results on the G+C content of M. leprae DNA presented in Table 2 concur with those of Imaeda et al. (19). However, the molecular weights of the M. leprae and M. vaccae genomes which we determined differ from those published by Imaeda et al. There were several differences in the way in which the reassociation studies presented in this study were conducted: (i) all DNA samples were sheared by sonication to yield fragments of 300 to 800 bp (determined by agarose gel electrophoresis); (ii) the DNA samples were denatured by maintaining the temperature in the spectrophotometer at 99°C for 20 min after the maximal hyperchromicity was achieved, and (iii) the $C_0t_{1/2}$ values were determined after the DNA was 50 to 65% renatured. In contrast, Imaeda et al., sheared some of their DNA by passage through a 26-gauge needle, which yields fragments of approximately 10 kb (average size) and some DNA by passage through a cell fractionator, which presumably yields smaller fragments. However, there is no indication of which method was used to shear specific DNAs. Imaeda et al. held the DNA samples at 96°C for 2 min, after maximal hyperchromicity had been achieved, which may be insufficient to completely denature DNA with G+C contents over 60%. Finally, these investigators determined their $C_0t_{1/2}$ values from

reaction rates observed during the first 15 min of the reaction. It has been pointed out by other investigators (33) that the reaction is not second-order during that period and thus may lead to erroneous calculations of genome size.

If the genome size of Corynebacterium 2628 LB reported by Imaeda et al. (19) is correct, then the genome of M. leprae is significantly different from this organism. Because of the disparities in genome sizes, it is very surprising that the Corynebacterium DNA showed 68% homology to M. leprae DNA as reported by those investigators.

Recombinant libraries representing greater than 99.99% of the genomes of M. leprae, M. vaccae, and M. "lufu" have been prepared by cloning into the cosmid vector, pHC79. The pHC79::M. leprae and pHC79::M. vaccae libraries were transduced into an in vivo packaging strain (E. coli χ2819) and were amplified, induced and stored at $4^{o}C$ as high titer phage lysates as described above. The pHC79::M. "lufu" libraries were transduced into E. coli χ2001 and stored frozen at $-70^{o}C$ as plasmid-containing cells. Fragments of M. leprae DNA subcloned from the recombinant cosmids into pYA626 can result in synthesis of polypeptides in minicells of E. coli K-12. Since DNA hybridization experiments using radioactively labeled fragments of inserted M. leprae DNA have demonstrated that these probes hybridize only to M. leprae chromosomal DNA, the unique polypeptides that are produced by minicells containing recombinant molecules must be encoded by the inserted M. leprae DNA. Two of the pYA626::M. leprae recombinant molecules which were analyzed produced more than one polypeptide, which could indicate that (i) some polypeptides may be

expressed from an M. leprae promoter or (ii) a polycistronic mRNA may be produced from the asd promoter or (iii) a newly synthesized polypeptide may be degraded by E. coli cellular proteases into one or several smaller stable polypeptides.

At present, there is no evidence that M. leprae promoters are functioning in E. coli K-12. We have recently obtained complementation of the gltA (citrate synthase) and aroB (dehydroquinate synthetase) mutations in E. coli by several different pYA626::M. leprae recombinant molecules but the M. leprae enzyme polypeptides are only expressed when the encoding DNA sequences are linked to the S. mutans asd promoter in one of e two possible orientations (W.R. Jacobs, M.A. Docherty, J.E. Clark-Curtiss, and R. Curtiss III, manuscript in preparation).

Experiments are in progress to further optimize expression of M. leprae genes in E. coli. This is being done to obtain expression of M. leprae genes specifying proteins that are potential targets for development of new anti-leprosy drugs. In addition, efficient expression of M. leprae gene products will facilitate immunological screening to identify protein antigens that might yield new diagnostic reagents or potential components of vaccines effective in preventing M. leprae infections. The recombinant molecules that have been shown to specify polypeptides by minicell analysis have been tested by an enzyme-linked immunosorbant assay (ELISA) with sera from eight leprosy patients (four borderline tuberculoid or tuberculoid and four borderline lepromatous or lepromatous patients; the sera were a gift from Dr. R. Gelber, Seton Medical Center, San Francisco, Calif.),

with monoclonal antibodies against specific M. leprae proteins (WHO-4, WHO-6, WHO-30, IIIE9, IIH9, E4/2 and IIC8; the monoclonal antibodies were gifts from Dr. J. Ivanyi, The Wellcome Foundation Ltd., Kent, England, and Dr. T.P. Gillis, National Hansen's Disease Center, Carville, La.), and with polyclonal anti-M. leprae serum prepared in rabbits (a gift from Dr. Gillis). To date, no positive ELISA reaction has been observed.

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
Escherichia coli K-12 χ289	F⁻ *tte-1*, prototroph	(11)
E. coli K-12 χ925	F⁻ *thr-1 ara-13 leu-6 azi-8*	Single colony
	tonA2 lacY1 minA1 glnV44 gal-6	isolate of P678-54
	λ⁻ *minB2 rpsL35 malA1 xyl-7*	(1)
	mtl-2 thi-1	
E. coli K-12 χ1849	F⁻ *tonA53 dapD8 minA1 purE41 glnV42*	(16)
	Δ40[*gal-uvrB*] λ⁻ *minB2 his-53 gyrA25*	
	metC65 oms-1 tte-1 Δ29[*bioH-asd*]	
	ilv-277 cycB2 cycA2 hsdR2	
	F⁻ Δ*araC766 tonA53 dapD8 proA370*	
E. coli K-12 χ2001	Δ*lacZ39 minA1* Δ69[*gal-chlD*] λ⁻ *tyrT58*	(7)
	Δ*galU183* Δ*trpE5 minB2 rfb-2 recA56*	
	relA1 Δ*thyA57 endA1 oms-1* Δ*asd-4*	
	rpoB402 cycB2 cycA1 hsdR2	

TABLE 1 (Continued)

E. coli K-12 χ2819	F⁻ *lacY1 glnV44 galK2 galT22* λ(*c1857 b2 red-3 S7*) *recA56* Δ*thyA57 metB1 hsdR2*	This paper
Mycobacterium leprae	Wild-type	Armadillo liver #29 infected with *M. leprae* pooled from seven patients; received from C. Shepard, Centers for Disease Control
M. "lufu"	Wild-type	J.K. Seydel, Institut Borstel
M. vaccae	Wild-type	C. Shepard, Centers for Disease Control
Micrococcus lysodeikticus	Wild-type	J. Lebowitz, University of Alabama in Birmingham
Pseudomonas aeruginosa PAO-2	Wild-type	J.B. Hansen, University of Alabama in Birmingham

TABLE 2. Base composition and genome sizes of DNA samples

DNA	Mole percent guanine + cytosine[a] by T_m	Mole percent guanine + cytosine[a] by buoyant density	Genome size[a] ($\times 10^9$)
Dasypus novemcinctus	35.7 ± 1 (2)[b]	36.2	ND[c]
Escherichia coli K-12 χ289	51.2 ± 0.6 (6)	49.0	2.5[d]
Micrococcus lysodeikticus	71.5 ± 1 (2)	72.6	ND
Mycobacterium leprae	56.2 ± 1 (3)	57.6	2.2 ± 0.3
M. "lufu"	61.3 ± 0.9 (3)	ND	3.1 ± 0.5
M. vaccae	65.3 ± 1 (3)	64.7	3.1 ± 0.8
Pseudomonas aeruginosa PAO-2	65.7 ± 1 (3)	66.1	ND

[a]Calculated from equations given in Materials and Methods; values represent the mean ± the standard deviation.

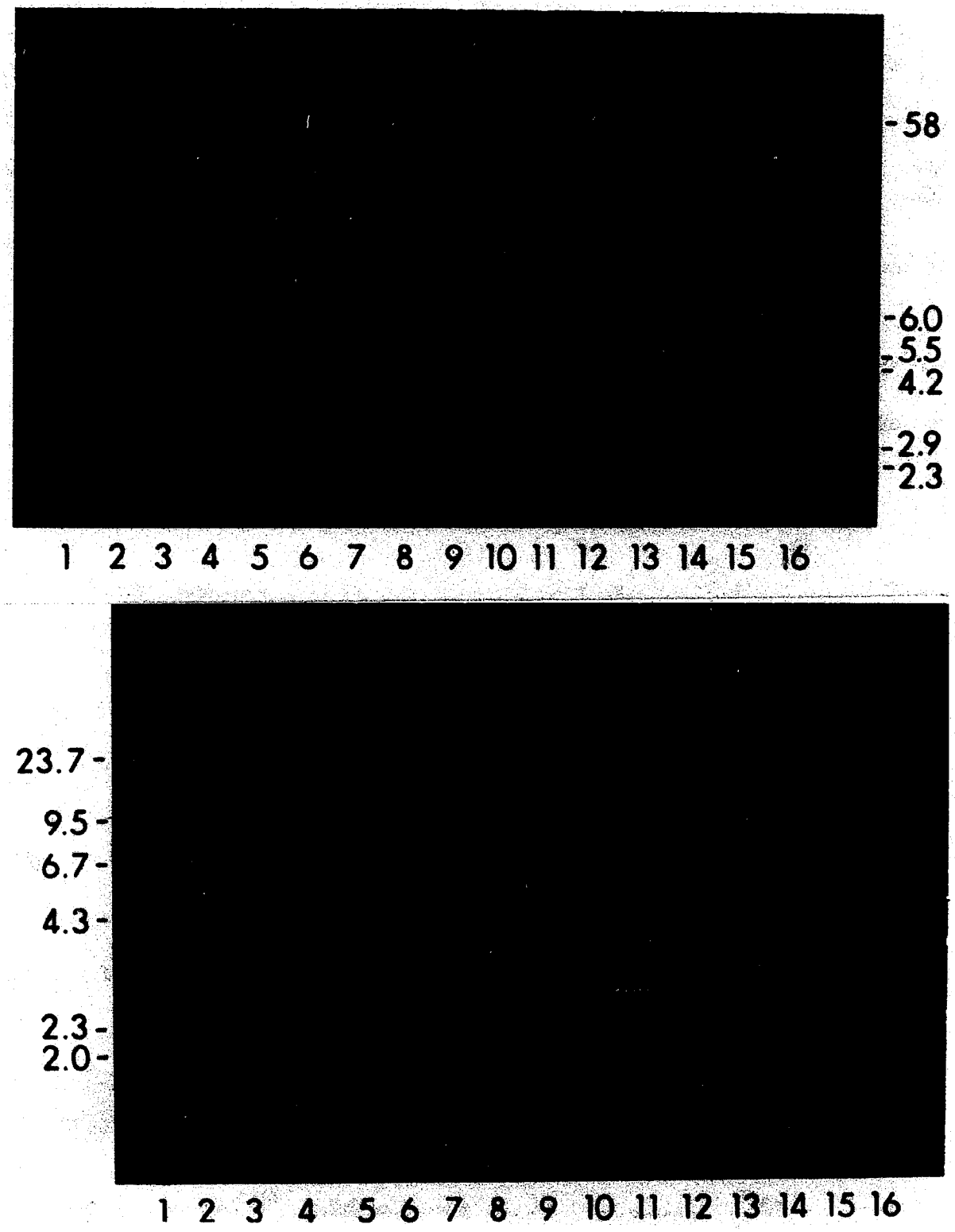
[b]Number of determinations.

[c]Not determined.

[d]Value from reference 5.

FIG. 1. Agarose gel electrophoresis of DNA from pYA626::*M. leprae* recombinant molecules before (A) and after (B) digestion with *PstI* restriction endonuclease. (A) Lanes 1 through 14 are, respectively: pYA626, pHC79, pYA1025, pYA1026, pYA1027, blank, pYA1028, pYA1029, pYA1030, pYA1031, pYA1032, pYA1033, pYA1034, pYA1035; Lane 15 is λ DNA digested with *HindIII* and Lane 16 is plasmid DNA from *E. coli* V517 (25), the sizes of which (in kb) are given to the right of the gel. (B) Lanes 1 and 16 are λ DNA digested with *HindIII* (the sizes of the fragments are given, in kb, to the left of the gel); Lanes 2 through 15 are, respectively: pHC79, pYA626, pYA1025, pYA1026, pYA1027, blank, pYA1028, pYA1029, pYA1030, pYA1031, pYA1032, pYA1033, pYA1034, pYA1035.

FIG. 1. (A, TOP; B, BOTTOM)



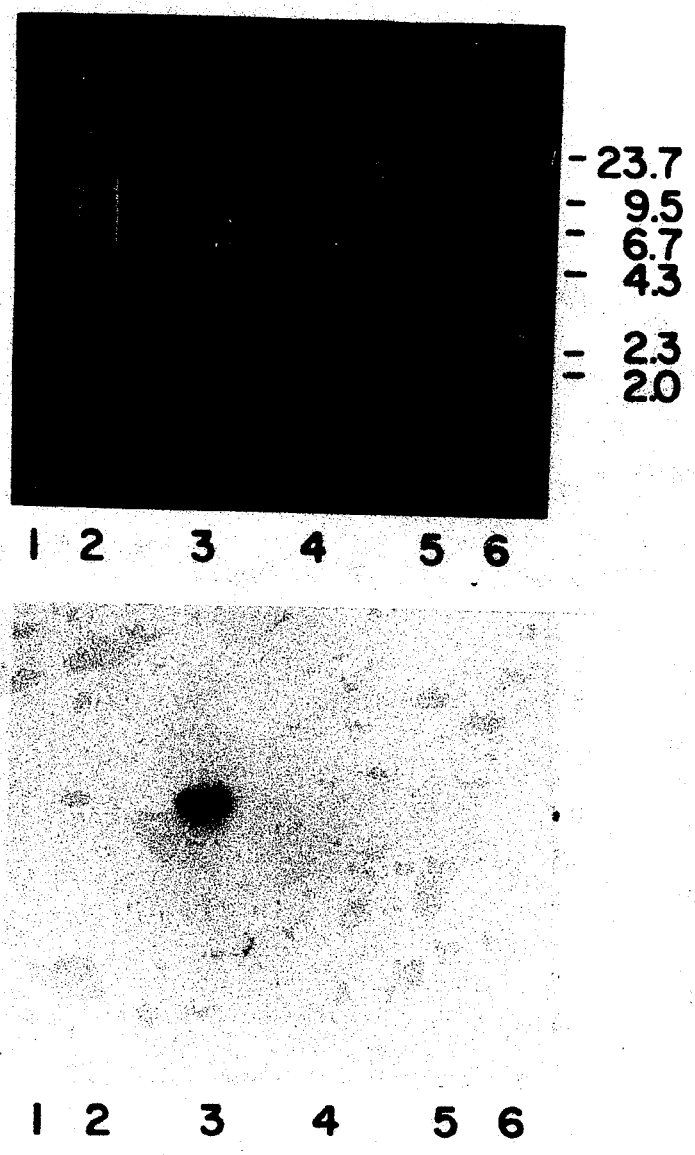
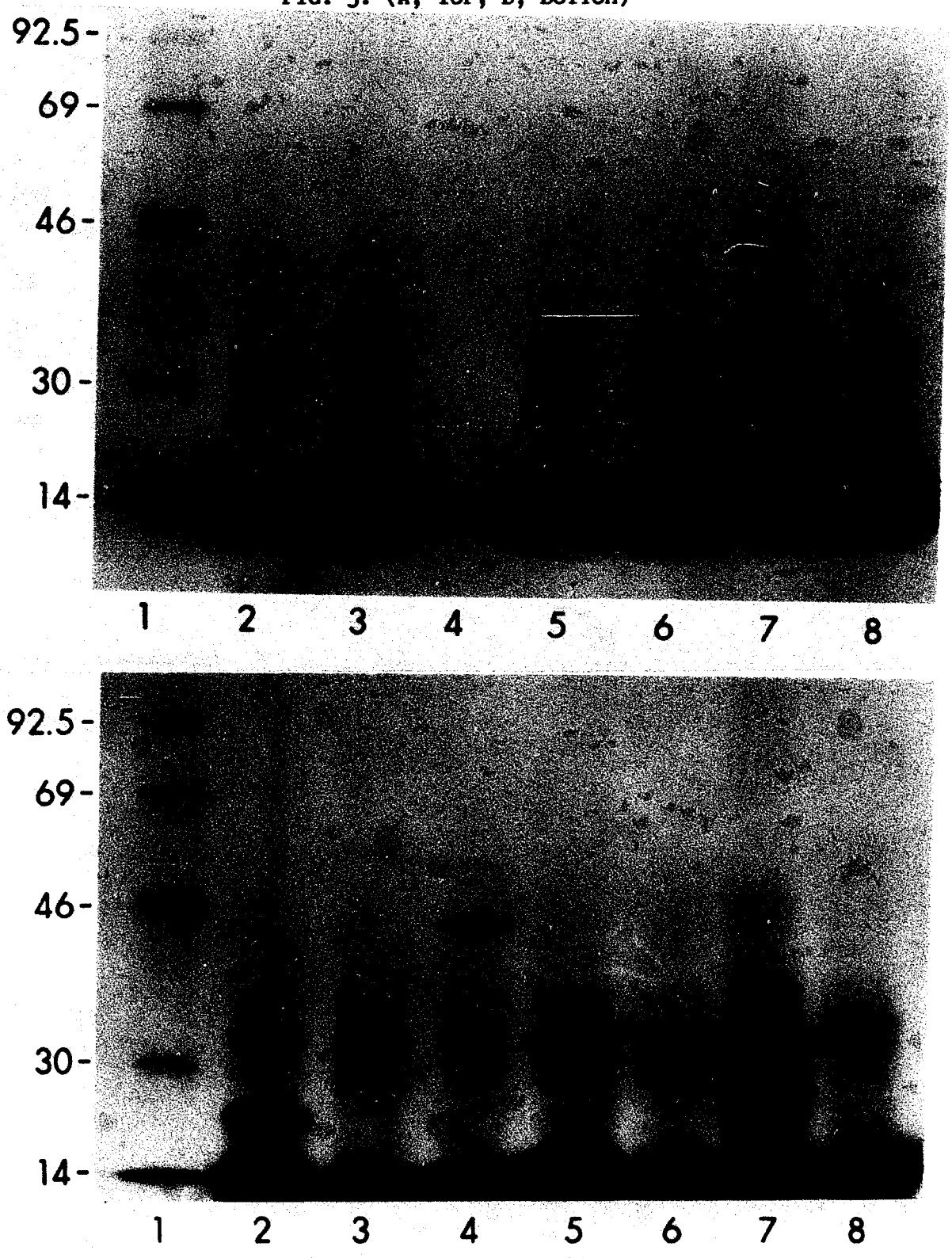


FIG. 2. Hybridization of ^{32}P-labeled M. leprae insert DNA from pYA1031 to PstI-digested chromosomal DNAs. (A) Photograph of the 0.7% agarose gel on which the chromosomal DNAs were separated; (B) Photograph of the autoradiograph of the hybridization. Lanes 1 through 5 contain completely digested chromosomal DNA from E. coli K-12, armadillo, M. leprae, M. "lufu", and M. vaccae, respectively. Lane 6 contains λ DNA digested with HindIII (the sizes of the fragments, in kb, are given to the right of the gel).

FIG. 3. SDS-PAGE analysis of ^{35}S-labeled polypeptides synthesized by minicells containing pYA626, pHC79, or pYA626::*M. leprae* clones. Lane 1 in both A and B contains ^{14}C-labeled protein standards (the sizes of which are given in kDa to the left of the gels); Lane 7 contains polypeptides synthesized by pHC79 and Lane 8 polypeptides synthesized by pYA626. (A) Lanes 2 through 6 contain polypeptides synthesized by pYA1028, pYA1029, pYA1025, pYA1034,and pYA1026, respectively; (B) Lanes 2 through 6 contain polypeptides synthesized by pYA1027, pYA1032, no plasmid, pYA1030, and pYA1031, respectively.

FIG. 3. (A, TOP; B, BOTTOM)



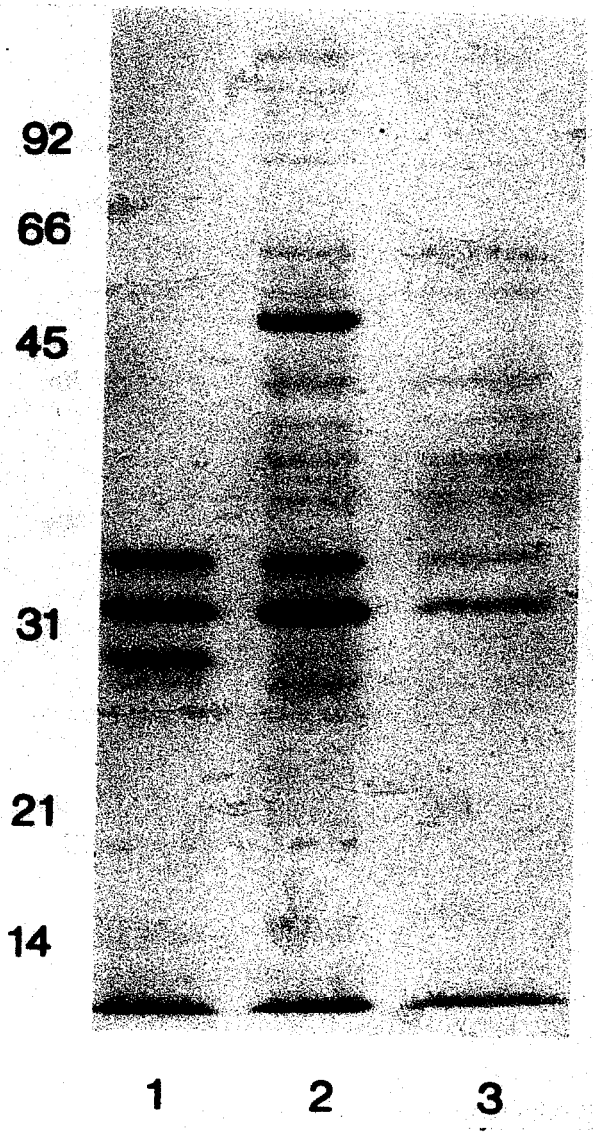


FIG. 4. SDS-PAGE analysis of ^{14}C-labeled polypeptides synthesized by minicells containing pBR322::*M. vaccae* recombinant molecules. Strain χ1849 containing pBR322 (lane 1), pYA1001 (lane 2), and pYA1002 (lane 3)The numbers to the left of the gel are the sizes (in kDa) of the ^{14}C-labeled protein standards separated by SDS-PAGE.

Classification: Microbiology

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

We had previously constructed plasmid and cosmid genomic libraries of Mycobacterium leprae, the causative agent of leprosy, but failed to observe complementation of auxotrophic mutations or mutations in carbohydrate catabolic pathways in Escherichia coli K-12 by cloned DNA. Genomic libraries of M. leprae DNA partially digested with PstI were subsequently constructed into 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 E. 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. SDS-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 into 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 that DNA fragment was cloned into the PstI site of pHC79, so as to allow expression from the β-lactamase promoter, the resulting recombinant failed to complement the mutation in the citrate synthase gene yet still produced the 46kDa polypeptide, but in fourfold less amounts than 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.

INTRODUCTION

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 1870's (2). Even so, M. leprae has been extremely difficult to study because of its inability to be cultivated in the laboratory. In the early 1960's, Shepard demonstrated the first successful cultivation of M. leprae in the footpads of mice (3). Significant quantities of the organism became available for subsequent research upon the discovery that M. leprae produced a systemic infection in the nine-banded armadillo (4,5).

We had previously screened genomic libraries of M. leprae DNA cloned into both plasmid and cosmid vectors and had failed to observe any complementation of a variety of mutations in amino acid, purine, and vitamin biosynthetic pathways or carbohydrate catabolic pathways in E. coli K-12 (6). We cloned M. leprae DNA into the expression vector pYA626 and were able to demonstrate the expression of M. leprae polypeptides in minicells containing recombinant M. leprae molecules (6). pYA626 contains the promoter of the aspartate β-semialdehyde dehydrogenase gene (asd) of S. mutans which has the unique structure of five overlapping and tandem Pribnow boxes and is very efficiently expressed in E. coli (6,7,Cardineau,G and Curtiss, R.III., manuscript submitted). In this manuscript we describe the

complementation of a mutation in the citrate synthase 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 strains used in this study. P1 transduction (15), cosmid transduction (6), and transformation (16) were performed as described previously.

Media. E. coli strains were grown in L broth (17) supplemented with diaminopimelic acid and thymidine, if necessary, or minimal salts broth or agar supplemented with amino acids, nucleotides, vitamins, glucose or other carbon sources (15).

Preparation of DNA. M. leprae, M. vaccae, M. "lufu", and Dasypus novemcinctus (nine-banded armadillo) DNAs were isolated and purified as described previously (6). Plasmid DNA was extracted according to the technique of Birnboim (18) with subsequent purification by centrifugation on cesium chloride-ethidium bromide density gradients, if necessary. Linear sucrose gradients were prepared by thawing Beckman polyallomer tubes containing 5.0 ml of frozen 20% sucrose in TE (10 mM Tris-HCl,pH 8.0;1.0 mM EDTA) at 4°C 2 h prior to use. Partially digested DNA was fractionated by layering 10 μg on a sucrose gradient, centrifuging the tubes at 45,000 rpm in a Beckman VTi65 rotor for 85 min at 15°C, puncturing the bottoms, and collecting drops by gravity flow. DNA was precipitated with yeast carrier RNA (Sigma), resuspended in water, mixed with vector, and ligated using T4 ligase. DNAs were analyzed on 0.7% agarose gels (19).

Enzymes. Restriction enzymes and DNA modifying enzymes were obtained from Bethesda Research Laboratories, New England Biolabs, or

Promega-Biotec and used according to the manufacture's recommendations. Calf intestine alkaline phosphatase was obtained from Sigma.

Minicell analysis. Minicells from 100 ml overnight cultures of χ925, χ2338, χ1488, or χ2874 containing various recombinant molecules were isolated and labelled as described previously (6). Radiolabelled proteins were resolved on 0.75mm thick by 20cm or 40cm long 7.5-15% SDS-polyacrylamide gels using the Laemmli buffer system (20), treated with Enhance (New England Nuclear), dried, and autoradiographed using Kodak X-Omat AR film. [^{14}C]-labelled molecular weight protein standards were obtained from Amersham.

Hybridization analysis. Probes were nick-translated using the nick-translation kit of Bethesda Research Laboratories. Southern hybridization analyses were performed using Genescreen (New England Nuclear), following the manufacturer's recommendations, in 1% SDS, 1.0M NaCl, and 10% dextran sulfate at 65^{0}C. Colony hybridization was performed as descibed by Maniatis et al (19). Autoradiographs were made on Kodak X-Omat AR film.

Citrate synthase assay. Cell extracts were prepared by sonicating mid log phase cells that had been grown in L broth supplemented with thymidine (and tetracycline for plasmid containing cells), washed with 0.1 M potassium-phosphate buffer (pH 7.0), and concentrated 50 fold in the phosphate buffer. The resulting suspension was pelleted at 100,000 x g to remove cell debris, and extensively dialyzed against 0.1 M potassium-phosphate buffer. The citrate synthase assay was performed as previously described (21). Oxaloacetate and acetyl CoA

were obtained from Boehringer Mannheim and 5,5'-dithiobis(2-nitrobenzoic acid) from Aldrich.

RESULTS

Cloning of an M. leprae gene that complements a mutation in the citrate synthase gene of E. coli. The expression vector pYA626 contains a 209 bp EcoRI-PstI fragment from the asd gene of S. mutans which replaces the 755 bp EcoRI-PstI fragment of pBR322. The S. mutans fragment contains the promoter region, the Shine-Dalgarno sequence, and the sequence encoding the 41-amino terminus amino acids of the asd gene, which is in phase with the 104 amino acids of the carboxy-terminus of the β-lactamase, as well as the unique PstI site downstream from the asd promoter. M. leprae DNA that had been partially digested with PstI was size fractionated on sucrose gradients. Fractions containing molecules of average size of 3 kb and 6 kb, respectively, were ligated to pYA626 that had been digested with PstI and treated with alkaline phosphatase. The resulting ligation mixture was transformed into χ2338 and the transformants were plated on L agar containing thymidine and tetracycline. 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 the citrate synthase (gltA), dehydroquinate synthetase (aroB), thymidylate synthetase (thyA), succinyl-diaminopimelate aminotransferase (dapD), and tryptophan biosynthetic (ΔtrpBC) genes of χ2338. Complementation of the gltA16 allele from each library was observed at a frequency of 10^{-4} and of the aroB mutation at a frequency of 10^{-7}. We did not observe complementation of any of the the other

mutations in this screening. Retransformation of χ2338 with plasmids isolated from clones that were selected for their ability to grow on minimal medium lacking glutamate in the presence of tetracycline showed a 100% cotransformation frequency of tetracycline-resistance and the gltA-complementing activity. The clones that complemented the gltA mutation were able to form colonies 1.0-2.0 mm in diameter on minimal medium lacking glutamate and containing tetracycline after four days growth at 37°C. Similar results were obtained when the clones were grown at 30°C.

Digestion of pYA1036 (the recombinant molecule complementing the citrate synthase gene from the 3 kb library) by the restriction nuclease PstI revealed a single 2.6 kb insert in pYA626 following agarose gel electrophoresis (Fig. 1B, lane 1). The gltA-complementing recombinant molecule found in the 6 kb library (pYA1037) also contained a 2.6 kb PstI fragment, plus four additional small PstI fragments (Fig. 1B, 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 both plasmids (Fig.1A and Fig. 1B).

Southern blot analysis demonstrated that the cloned gltA-complementing DNA fragment hybridized very strongly to M. leprae chromosomal DNA and hybridized weakly to unique PstI fragments of the two other mycobacteria (Fig.2). 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 our previously described *M. leprae* DNA cosmid libraries constructed in pHC79 (6) for the presence of sequences that hybridize with the 2.6kb *gltA*-complementing fragment. χ2819 was transduced with lysates of *in vivo* packaged recombinant cosmid molecules, tetracycline-resistant colonies were selected, and colony hybridizations were performed (Fig. 3). Strong hybridization with 20 out of 1600 colonies tested was observed, thus confirming the presence of the *gltA* complementing fragment in our original libraries, although we had failed to observe complementation of the *gltA* mutation in χ2338, probably because the *M. leprae* promoter is not expressed 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, the 2.6 kb *gltA*-complementing fragment was recloned into pYA626 with screening for recombinant molecules containing the fragment in either orientation with respect to the *asd* promoter. The recombinant molecule pYA1040 which was reconstructed tohave the 2.6 kb fragment in the same orientation as in pYA1036 was able to complement the mutation in the citrate synthase gene of χ2338. However, when the 2.6kb 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.4).

In order to determine what polypeptides were being synthesized by the cloned DNA, radiolabelled polypeptides produced in minicells containing the various recombinant plasmids were analyzed. The

vector, pYA626, specified the production of the 14 kDa fusion polypeptide of the aspartate β-semialdehyde dehydrogenase and the β-lactamase, as well as the 34 kDa tetracycline-resistance gene product (Fig.5, 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 which is not produced in minicells containing pYA1041 (Fig.5, lanes 3,4,5, & 6). In fact, pYA1041 the non-complementing clone which has the 2.6 kb insert backwards relative to the asd promoter specifies the production of two different polypeptides of 14 and 25 kDa (Fig.5, lane 5). Neither of these polypeptides are specified by any of the gltA-complementing recombinant molecules.

To test whether the 46kDa gltA-complementing polypeptide was a fusion polypeptide with the amino terminus of the aspartate β-semialdehyde dehydrogenase gene product, the 2.6kb PstI fragment was inserted into the PstI site of pHC79 (22), which was used as a plasmid vector for this experiment. Both possible constructs 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 the gltA-complementing molecules with respect to the β-lactamase promoter) nor pYA1045 were able to complement the gltA mutation in χ2338 (Fig. 4) If the gltA-complementing polypeptide was fused with the 41 amino acid terminus of the asd gene product, it should also form a fusion polypeptide with the 159 amino acid terminus of β-lactamase in pHC79 producing a much larger fusion polypeptide (23). Both pYA1036 and

pYA1044 specified the production of a 46 kDa polypeptide (Fig. 6A, lanes 3 & 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 tetracycline-resistance gene product (Fig. 6B). Minicells isolated from χ2338 containing pYA1041 only produced a polypeptide of 14 kDa and not the 25 kDa polypeptide that was observed in χ925 minicells containing pYA1041. The presence of an amber suppressor mutation (glnV44) in χ925 that is not in χ2338 might account for this difference. This same 14 kDa polypeptide is not specified by pYA1045, but rather a unique polypeptide of 32 kDa is produced, suggesting the production of a fusion polypeptide in each case.

Citrate synthase catalyzes the condensation of an acetyl group from Acetyl CoA to oxaloacetate to form citrate and CoASH (21). Citrate synthase assays were performed on cell extracts of χ2338; χ2339, a Glt$^+$ transductant of χ2338; and χ2338 cells containing pYA626 or pYA1036; using an assay that measures CoASH formation (21). Extracts from χ2338, which contained the gltA16 allele, demonstrated no detectable citrate synthase activity whereas the P1 transductant carrying the wild type allele did (data not shown). We failed to observe activity in either extract containing pYA626 or pYA1036.

Stability of the gltA-complementing polypeptide. A pulse-chase analysis was performed using two isogenic strains of minicells which only differ by the presence of lon$^+$ or lon-9 alleles to determine the stability of the gltA-complementing polypeptide. The half-lives of the 46 kDa polypeptide and the tetracycline resistance gene product

were 45 minutes, based on densiometric scans of the autoradiographs of polypeptides observed after 60 min incubation (Fig. 7). However, the rate of degradation did not appear to be constant, and the half life of the 46 kDa protein appeared to be as low as 17 min when calculated on the basis of the observed in the first 20 min. Since the product of the lon gene is known to be a protease responsible for the degradation of abnormal proteins in E. coli (24,25,26,27,28), a pulse-chase analysis comparing minicells isolated from strains with a lon-9 or Lon^+ allele containing pYA1036 was performed. No difference was observed in the rates of degradation of the gltA-complementing polypeptide between the two strains.

DISCUSSION

The results demonstrate the expression of a *M. leprae* gene that complements a mutation in the citrate synthase gene in *E. coli* K-12. The complementing DNA fragment specifies the production of a 46 kDa polypeptide when expressed from an external promoter. We had been unsuccessful in observing complementation of a variety of mutations in *E. coli* by screening genomic libraries of cloned *M. leprae*, *M. vaccae*, and *M. "lufu"* DNAs (6).

There are numerous possibilities for the failure to express foreign genes in *E. coli*. It is quite likely that mycobacterial promoters are not expressed or are expressed inefficiently by *E. coli* transcriptional machinery. It is unknown whether the *M. leprae* promoter for the citrate synthase-complementing gene is present within the cloned DNA fragment, but the failure to observe complementation by cosmid clones known to contain a sequence that hybridizes with the citrate synthase-complementing fragment supports both hypotheses. Since the 46 kDa polypeptide is synthesized under the control of the β-lactamase promoter as well as the *asd* promoter, this polypeptide is not a fusion polypeptide. Therefore, *M. leprae* translation initiation sites are being recognized by *E. coli* translational machinery, although the efficiency of this recognition might not be optimal. Instability of the foreign gene product may be a result of the *E. coli* recognizing the protein as foreign and degrading it. The *lon* gene encodes one such protease which is known to degrade abnormal proteins, including fusions of β-galactosidase with foreign gene

products (29). No evidence was found to implicate the lon gene product as being responsible for the degradation of the 46 kDa polypeptide. Mosteller et. al. (30) found that the apparent half lives of unstable E. coli proteins in exponentially growing cultures to be 2 to 23 h. Since the apparent half life of the 46 kDa polypeptide is 45 min or less it is possibly being recognized as foreign, and consequently being degraded. A mutation in this protein degradation system should result in a stabilization of the 46 kDa polypeptide. Since expression of the 46 kDa polypeptide by the β-lactamase promoter is insufficient to detect complementation of the citrate synthase mutation, mutagenesis of χ2338 containing pYA1044, followed by selection for complementation, may result in the selection of protease-deficient mutants.

A mutation in the citrate synthase gene of E. coli results in auxotrophy for the glutamate family of amino acids (31,32). The complementation of the gltA16 mutation observed is most likely the result of cloning of the M. leprae citrate synthase gene. The molecular weight of the cloned E. coli citrate synthase gene has been determined to be 46,000 by SDS-polyacrylamide gel electrophoresis (33), although the predicted molecular weight inferred from the DNA sequence is 48,069 (34).The molecular weight of the M. leprae citrate synthase from cell extracts has not yet been determined . Initial attempts to demonstrate citrate synthase activity in extracts from cells containing the gltA-complementing activity have been unsuccessful. Failure to observe complementation of the citrate synthase mutation by the cloned gene when expressed from the

β-lactamase promoter indicates that low activity is inadequate for complementation. Wheeler has demonstated low citrate synthase activity from extracts of M. leprae cells grown in armadillos, but high levels of oxaloacetate are required to detect activity (35). This suggests that the Km's for oxaloacetate are different or that the conditions for optimal in vitro activity for the two enzymes are considerably different. Citrate synthase is a key regulatory enzyme of the Kreb's cycle and is regulated differently in another obligate intracellular parasite, Rickettsia prowazekii (36), and in mycobacteria(37) compared to the E. coli citrate synthase. Altered regulatory effects could also account for low activity in vivo.

The ability to express M. leprae genes in E. coli will provide many new opportunities to understand the disease of leprosy. The expression of protein antigens of M. leprae should allow for the dissection of those entities that are important for protective immunity as well as provide a source of material for a possible vaccine. Young et. al. have recently demonstrated the expression of M. leprae polypeptide antigens fused to β-galctosidase using a λgt11 library (manuscript submitted). The ability to complement a mutation in E. coli demonstrates the expression of a functional M. leprae enzyme which is not a fusion protein. The use of promoter expression vectors may enhance expression and minimize proteolytic breakdown observed with protein fusions. Promoter expression vectors can also be used for expression of proteins that are targets of anti-leprosy drugs providing a source of material to determine the mechanisms of actions of such drugs. The expression of

dihydropteroate synthetase, the putative site of action of the anti-leprosy drug dapsone, might not only explain its mechanism of action, but also supply clues to circumvent the serious problem of dapsone-resistant leprosy. The number of dapsone-resistant leprosy cases is increasing yearly and poses a serious problem to developing nations (38).

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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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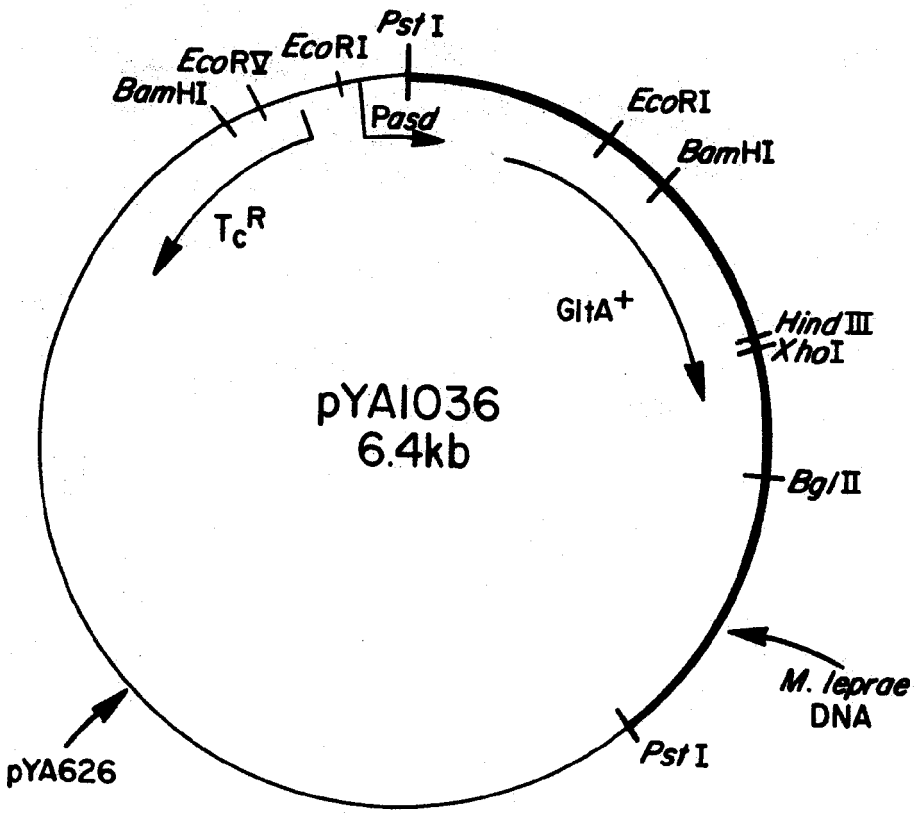
TABLE 1. Bacterial Strains

Strain	Genotype	Source
χ925	F⁻ thr-1 ara-13 leu-6 azi-8 fhuA2 lacY1 minA1 glnV44 gal-6 λ⁻ minB2 rpsL135 malA1 xyl-7 mtl-2 thi-1	(8)
χ1488	F⁻ minA1 purE41 glnV42 λ⁻ pdxC3 minB2 his-53 metC65 rpsL97 tte-1 xyl-14 ilv-277 cycB2 cycA1 hsdR2	(9)
χ2338	F⁻ ΔaraC766 fhuA53 dapD8 minA1 gltA16 Δ69[gal-chlD] λ⁻ ΔtrpBC13 minB2 rfb-2 gyrA25 ΔthyA57 endA1 oms-1 aroB15 cycB2 cycA1 hsdR2	17 steps from χ1776 (10)
χ2339	F⁻ ΔaraC766 fhuA53 dapD8 minA1 Δ69[gal-chlD] λ⁻ ΔtrpBC13 minB2 rfb-2 gyrA25 ΔthyA57 endA1 oms-1 aroB15 cycB2 cycA1 hsdR2	P1L4(χ2491) → χ2338, selection for Glt⁺
χ2819	F⁻ lacY1 glnV44 galK2 galT22 λ (cI857 b2 redβ3 S7) recA56 ΔthyA57 metB1 hsdR2	(6)
χ2844	F⁻ tsx-462::Tn10 λ⁻	Selected as a T6^R, Tc^R transductant of MG1655(11) using λ561(12)
χ2845	F⁻ ara-14 leu-6 azi-6 fhuA29 lacY1 tsx-462::Tn10 lon-9 purE42 glnV44 galK2 λ⁻ trpE38 lpcB rpsL73 xyl-5 mtl-1 thi-1	P1(χ2844) → RGC103 (13)
χ2874	F⁻ minA1 tsx-463 lon-9 purE41 glnV42 λ⁻ pdxC3 minB2 his-53 metC65 rpsL97 tte-1 xyl-14 ilv-277 cycB2 cycA1 hsdR2	P1(χ2845) → χ1488; selection of fusaric acid resistance (14)

Fig. 1. Restriction analysis of gltA-complementing clones. (A) Restriction map of plasmid pYA1036. Arrows indicate the direction of transcription for genes on this plasmid. (B) Ethidium bromide-stained agarose gel. Lanes: MW, HindIII-digested λ DNA and HaeIII-digested ϕX174 DNA; 1, pYA1036 digested with PstI; 2, pYA1037 digested with PstI; 3, pYA1036 digested with BamHI; 4, pYA1037 digested with BamHI; 5, pYA1036 digested with EcoRV and XhoI; 6, pYA1037 digested with EcoRV and XhoI.

Fig. 1. (A, TOP: B. BOTTOM)



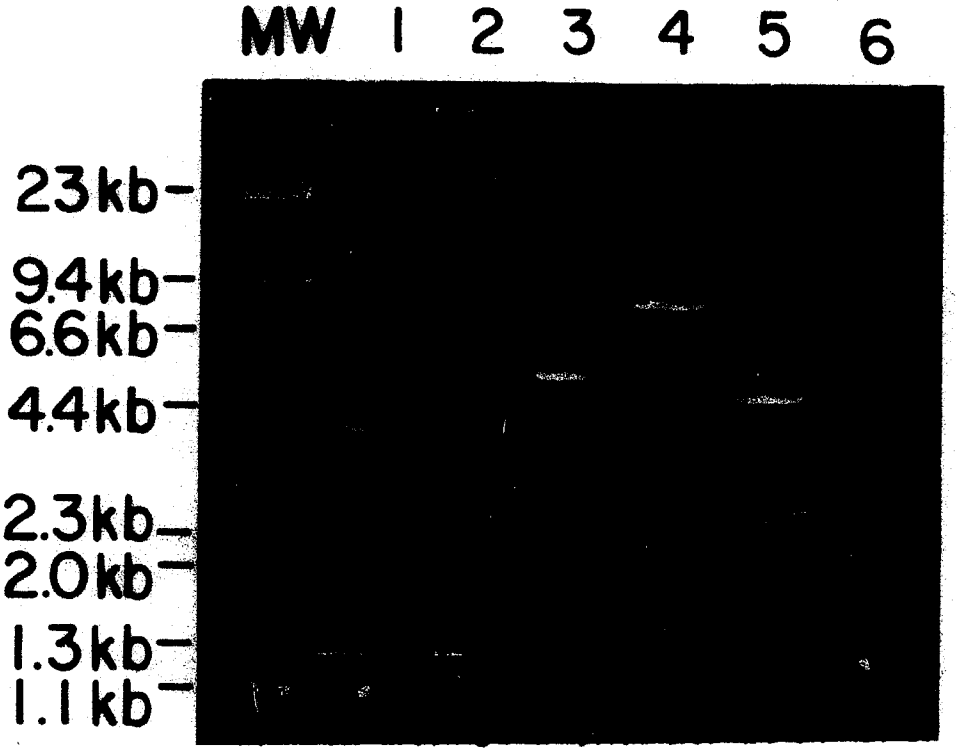
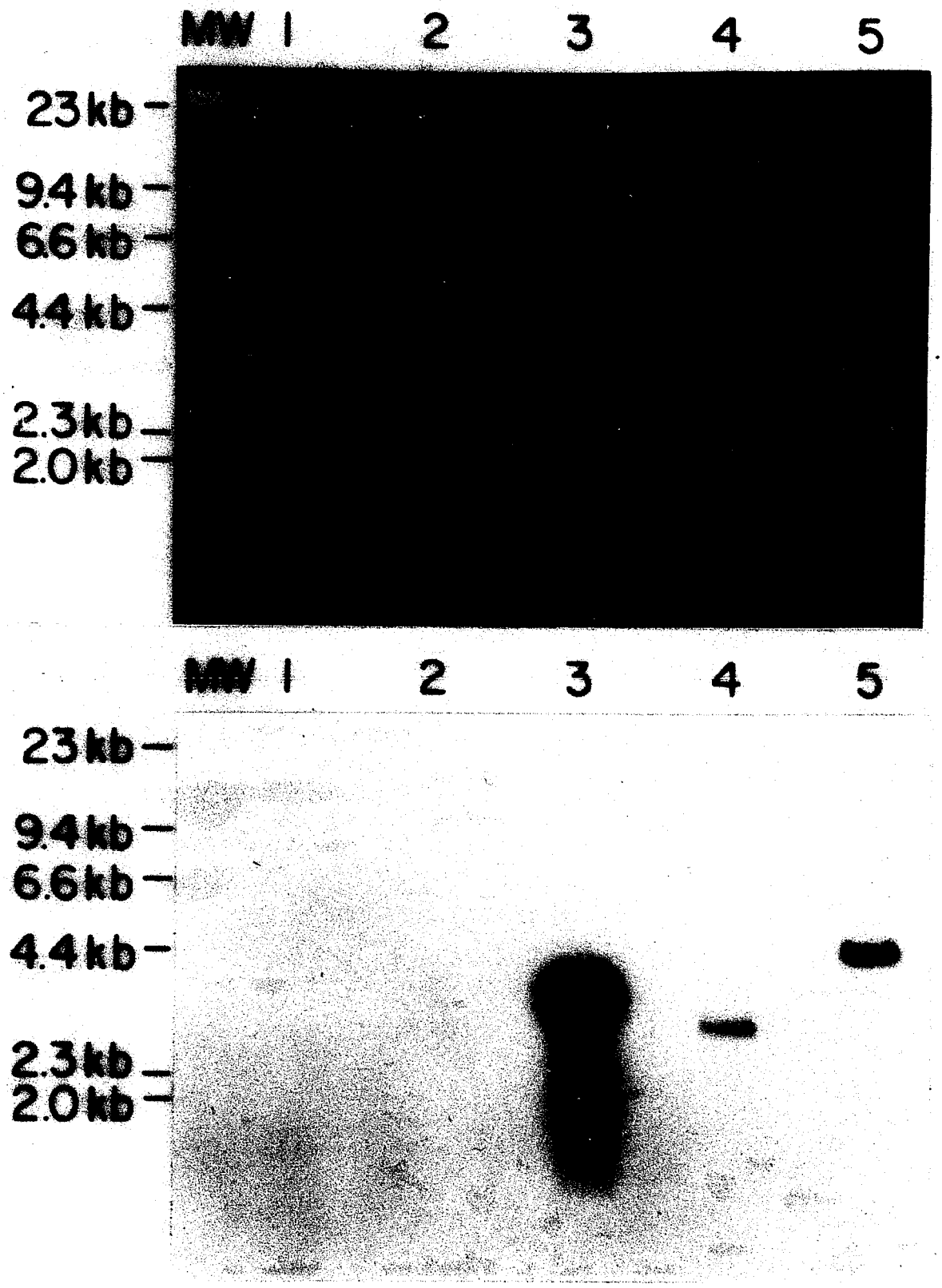


Fig. 2. Southern hybridization of the 2.6 kb gltA-complementing DNA fragment to various chromosomal DNAs. (A) Ethidium bromide-stained 0.7% agarose gel in which one microgram of various chromosomal DNAs totally digested with PstI were electrophoresed and transferred to GeneScreen (NEN). (B) Autoradiogram of the blotted chromosomal DNA probed with the 2. 6kb PstI gltA-complementing DNA fragment that had been labelled by nick translation with [^{32}P]. Lanes: MW, 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.

Fig. 2. (A, TOP; B, BOTTOM)



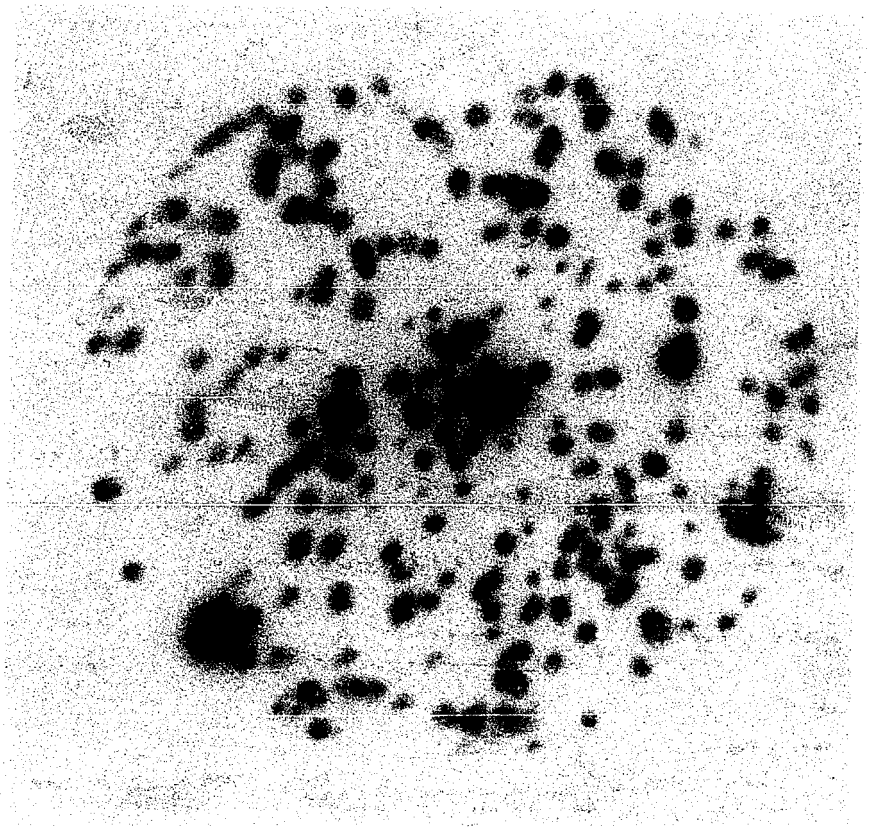


Fig. 3. Colony hybridization of M. leprae cosmid clones with the gltA-complementing DNA fragment. χ2819 was transduced with tetracycline-resistant particles from lysates prepared by inducing in vivo packaging strains containing pHC79::M. leprae cosmid molecules (6) and diluted to yield roughly 200 tetracycline-resistant colonies on an L agar plate containing tetracycline. The colonies were grown to 0.5-1.0mm in diameter, transferred to nitrocellulose, lysed, baked, and probed with the 2.6 kb PstI gltA-complementing DNA fragment that had been labelled by nick translation with [^{32}P].

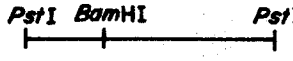
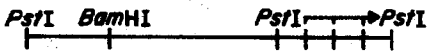
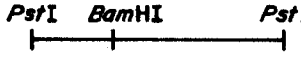
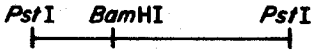
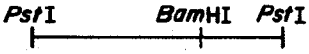
RECOMBINANT MOLECULE	VECTOR	INSERT DESCRIPTION	Glt PHENOTYPE
pYA1036	pYA626		Glt+
pYA1037	pYA626		Glt+
pYA1040	pYA626		Glt+
pYA1041	pYA626		Glt-
pYA1044	pHC79		Glt-
pYA1045	pHC79		Glt-

Fig. 4. 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. The 2.6 kb-PstI-gltA-complementing M. leprae DNA fragment was recloned into the PstI site of pYA626 or pHC79. The schematic representation of the fragment displays its orientation with respect to the asd or β-lactamase (bla) promoters. The recombinant plasmids were transformed into χ2338, selecting for tetracycline resistance and screened for their ability to complement the gltA mutation.

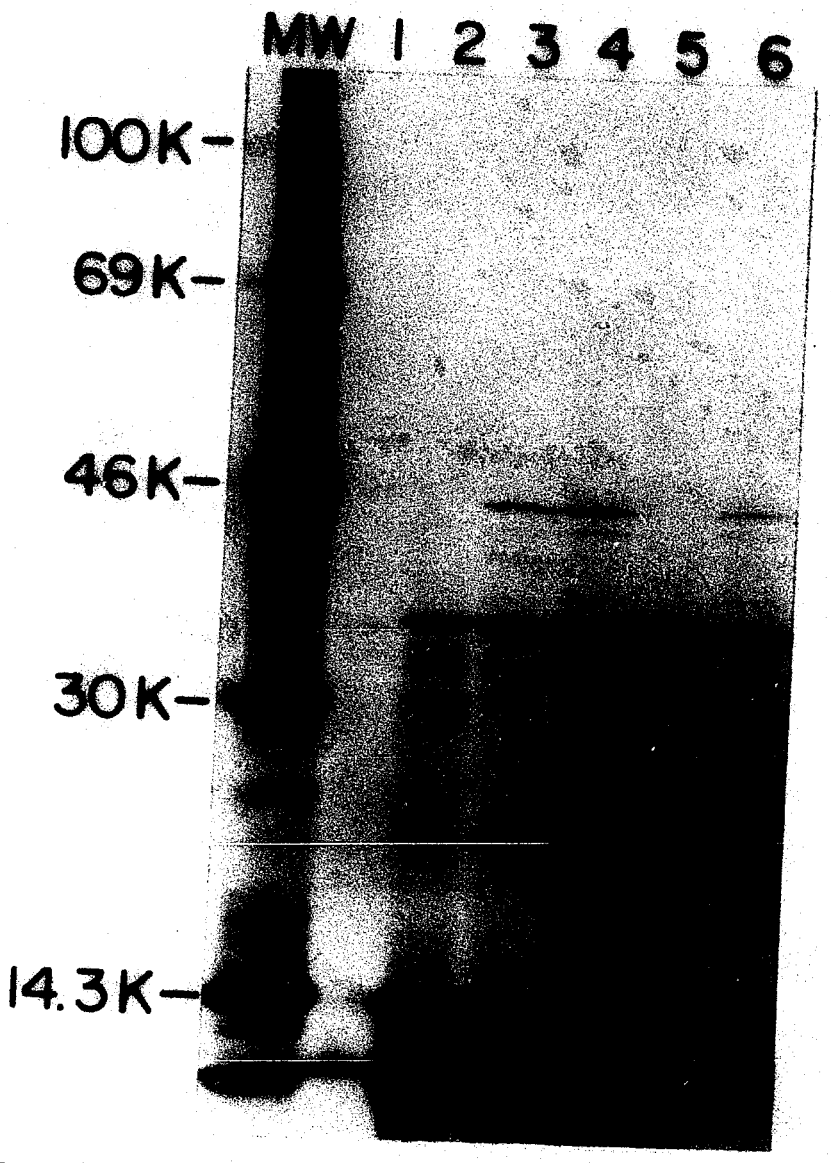


Fig. 5. Fluorograph of polypeptides produced in minicells containing gltA-complementing and non-complementing recombinant molecules. Minicells were isolated from χ925 that had been transformed with various pYA626::M. leprae recombinant molecules. The isolated minicells were labelled with [^{35}S]-methionine, lysed, and run on a 7.5-15.0% SDS-polyacrylamide gel. The gel was treated with Enhance (NEN), dried, and exposed to x-ray film; the resulting fluorograph is shown. Lanes: MW, [^{14}C]-labelled 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. 6. Expression of the gltA-complementing polypeptide from the asd or bla promoters. Minicells were isolated from χ2338 clones containing various recombinant molecules, labelled with [^{35}S]-methionine, lysed, run on a 7.5=15.0% SDS-polyacrylamide gel, and fluorography was performed. (A) The resulting fluorograph. Lanes: 1, χ2338 minicells containing pYA626; 2, χ2338 minicells containing pHC79; 3, χ2338 minicells containing pYA1036; 4, χ2338 minicells containing pYA1041; 5, χ2338 minicells containing pYA1044; 6, χ2338 minicells containing pYA1045. Densitometric scans of lane 3 (B) and lane 5 (C) of the putative citrate synthase and the tetracycline-resistance gene product were performed on the fluorograph.

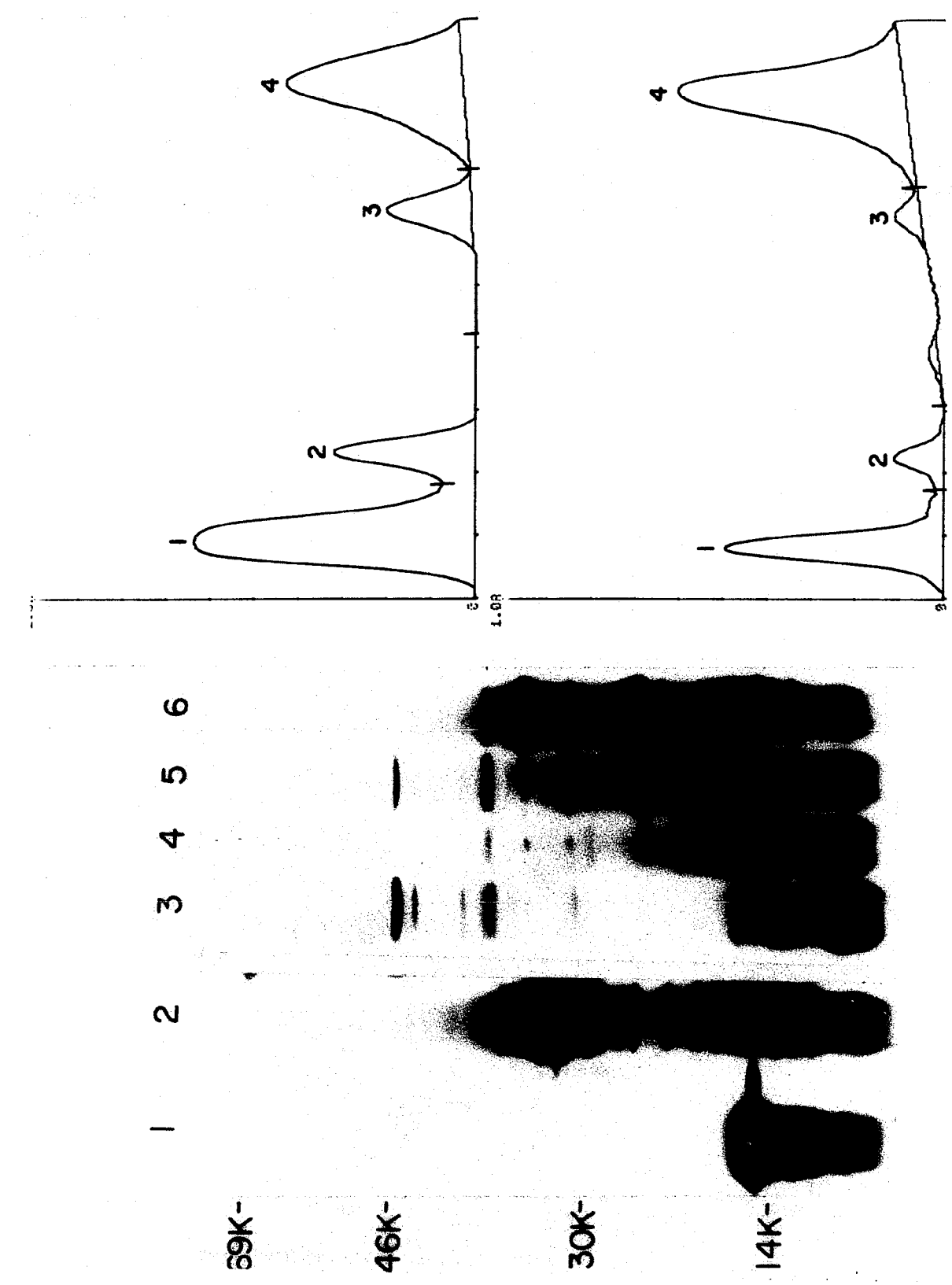


Fig. 6 (A,LEFT; B,RIGHT)

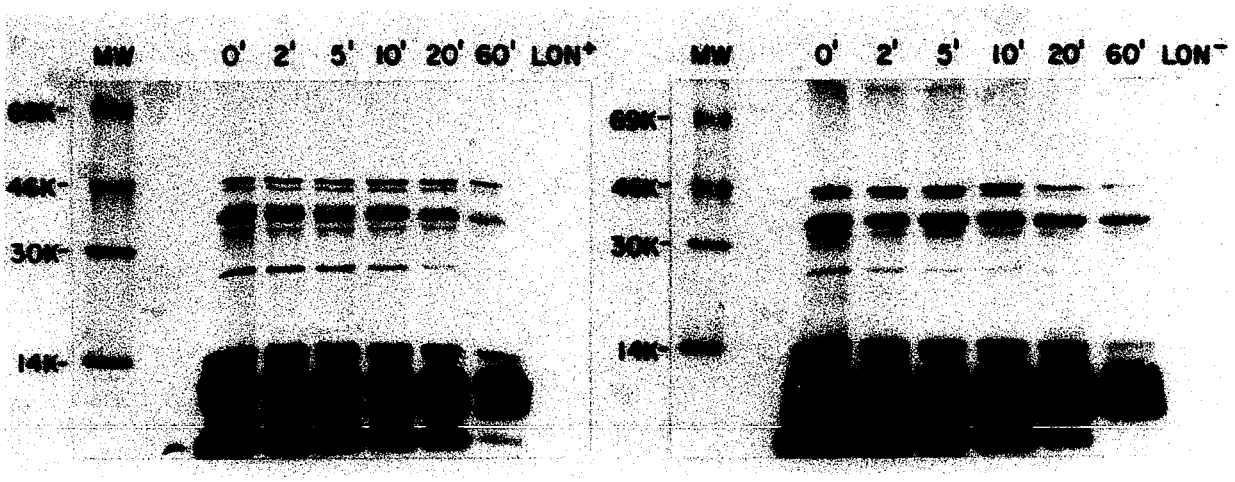


Fig. 7. Effect of the lon gene product on the stability of the M. leprae gltA-complementing polypeptide expressed in E. coli. Minicells containing pYA1036 were isolated from either χ1488 or χ2874, labelled for two minutes with 10 μCi [^{35}S]-methionine, and chased with 1000 molar excess cold methionine. Samples were removed at 0, 2, 5, 10, 20, or 60 min after the addition of excess methionine, lysed, run on a 20cm 7.5-15.0% SDS-polyacrylamide gel, and fluorography was performed.

SUMMARY

There are 4 to 10 new cases of leprosy reported every year for every 1000 people in areas of the world where leprosy is endemic. Victims are ostracized and 20 to 30% suffer crippling deformities of the hands and feet (1). A major goal of the World Health Organization is to control and eradicate this disease. The construction of genomic Mycobacterium leprae libraries and expression of that DNA in Escherichia coli K-12 will play an important role in achieving that goal.

One of the ways that the control of leprosy could be more effective is by having a means for early detection of infection with M.leprae. Specific DNA sequences that are unique to M. leprae could be used as probes for early diagnosis of the disease. We have been screening 2 to 5 kb inserts in the plasmid genomic libraries of M. leprae DNA by Southern blot analysis for fragments of DNA that only hybridize to M. leprae genomic DNA and fail to hybridize to other mycobacterial genomes. These probes could also be used to determine if the bacillus that causes lepromatous leprosy is different from the bacillus that causes tuberculoid leprosy by performing restriction fragment length polymorphism (RFLP) studies. Total chromosomal DNA from the two different isolates would be digested with various restriction enzymes, the resulting fragments would be separated on agarose gels, blotted to pieces of nitrocellulose, and then hybridized with 10 probes that had

been labelled with ^{32}P. If the isolates were identical strains, then the banding patterns of the hybridized DNA would be identical. If the patterns were different, it would suggest that there were differences in the DNA sequences due to mutations arising during the evolution of the organisms and that the isolates are therefore different. In either case, further investigation to determine what genetic factors of M. leprae play a role in the manifestations of the leprosy disease would be warranted. Natural infections of the armadillo (7), chimpanzee (2), and mangabey monkey (4) have been reported. It would be of interest to determine if these isolates varied as well to determine if there are any natural reservoirs of M. leprae infections.

Our work has demonstrated that a functional M. leprae enzyme can be expressed in E. coli. The potential exists that any M. leprae enzyme could be expressed in E. coli as long as the cloned M. leprae DNA was positioned so as to be expressed from a strong promoter. Cloning into pYA626 was limited by the fact that the only useful restriction site downstream from the asd promoter was PstI. pYA804 is a newly constructed derivative of pYA626 into which synthetic oligomers were inserted to introduce unique AsuII, XhoI, and HpaI restriction sites. AsuII is a particularly useful restriction site for efficiently constructing random genomic libraries, as it generates a two base-pair guanine-cytosine overhang that is compatible with overhangs generated by three different four base-pair cutting enzymes (TaqI, HpaII, and HinPI). In other words, genomic libraries could be generated by digesting chromosomal DNA with any of the three four

base-pair cutting enzymes and then ligating the chromosomal fragments to the AsuII site of pYA804. The synthetic oligomer also includes a BamHI site and a SalI site, so that an EMBL4 lambda (3) derivative could be constructed containing the asd promoter. This lambda expression vector would allow the cloning of foreign DNA into BamHI, SalI or XhoI sites of the lambda phage and expression of genes from the asd promoter.

Prior to the development of dapsone in the early 1940's, there was no effective treatment for leprosy. Dapsone is a sulfone that is thought to affect folate metabolism of M. leprae by inhibiting dihydropteroate synthetase (6). Dapsone has been the drug of choice for treating leprosy because of its low cost, non-toxicity, and effectiveness against M. leprae. Since 1964, individual cases of dapsone-resistant leprosy have been documented and are reportedly increasing in countries where leprosy is endemic (5). The expression of a cloned M. leprae dihydropteroate synthetase in E. coli would permit the production of sufficient enzyme to rigorously study the effect of dapsone on its activity and to enable the isolation of mutations in the cloned gene that result in dapsone resistance. Such studies might suggest ways of combatting dapsone resistance. The expression of enzymes in E. coli that are potential target sites for other anti-leprosy drugs would allow a more rapid evaluation of their potential effectiveness.

Another major effort of the World Health Organization to prevent leprosy infections, is the development of a vaccine for prophylactic use. The ability to express various proteins from M. leprae in E.

coli offers a new source of antigens which can be studied to determine if they provide protective immunity to M. leprae infections. Young et. al., using the λgt11 expression system, have identified several protein fusions of β-galactosidase and M. leprae polypeptides that react with various monoclonal antibodies prepared against M. leprae cells (manuscript submitted). Similar studies using the EMBL4-asdP expression vector are underway.

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