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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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Strains of *Escherichia coli* K-12 were constructed that permitted the amplification of in vitro-packaged recombinant cosmid-transducing particles by in vivo repackaging of recombinant cosmid molecules. Thermal induction of these thermoinducible, excision-defective lysogens containing recombinant cosmid molecules yielded high titers of packaged recombinant cosmids and low levels of PFU. These strains were used to amplify packaged recombinant cosmid libraries of *Mycobacterium leprae*, *Mycobacterium vaccae*, *Salmonella typhimurium*, and *Streptococcus mutans* DNA. Contiguous and noncontiguous libraries were compared for the successful identification of cloned genes. Construction of noncontiguous libraries allowed the dissociation of desired genes from genes that were deleterious to the survival of a cosmid recombinant and permitted selection for unlinked traits that resulted in a selected phenotype. In vivo repackaging of recombinant cosmids permitted (i) amplification of the original in vitro-packaged collection of transducing particles, (ii) storage of cosmid libraries as phage lysates, (iii) facilitation of complementation screening, (iv) expression analysis of repackaged recombinant cosmids after UV-irradiated cells were infected, (v) in situ enzyme or immunological screening, and (vi) facilitation of recovery of recombinant cosmid molecules containing transposon inserts.

The technique of cosmid cloning (11) has provided an efficient means of cloning segments of DNA as large as 45 kilobase pairs (kbp) (30). Cosmids are plasmid cloning vectors that contain 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.

Analyses of recombinant DNA libraries for expression 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 permissible under the guidelines of the National Institutes of Health 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 to use 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 constructed a set of thermoinducible lysogens of *E. coli* K-12 containing a prophage that was defective in both excision and recombination functions. When these strains (containing recombinant cosmid molecules) were induced, they yielded high titers of transducing particles containing the recombinant cosmid molecules and low titers of infectious phage. The original cosmid library was thus amplified as a phage lysate of transducing particles. We have previously described the construction and amplification of *Mycobacterium leprae* cosmid library (9). In this report we further describe the construction of additional in vivo cosmid-packaging strains and demonstrate their usefulness in the analyses of cosmid libraries of *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 *c*I857 *b*2 *red*β3 *S*7, λ *h*φ80 Δ*att c*, and λ *b*2 *c* were obtained from Nat Sternberg. λ *c*I857 *b*2 *red*β3 was isolated as a spontaneous revertant of λ *c*I857 *b*2 *red*β3 *S*7 on SC180 (suppressor-free *E. coli* K-12 strain from O. Reyes). λ *vir* came from this laboratory. The strains were grown in 1% tryptone–0.5% yeast extract–0.5% NaCl–0.4% maltose (TYM broth), prior to infection by λ or by cosmid-transducing particles, and in 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) were supplemented with 0.5% carbohydrates and amino acids, purines, pyrimidines, and vitamins at concentrations described previously (13). Eosin

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
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* λ⁻ *galK2 galT22 tyrT58 metB1 hsdR514 trpR55*	Philip Leder[a]
χ2001	F⁻ Δ*araC766 fhuA53 dapD8 proA318 minA1* Δ69(*gal-chlD*) λ⁻*tyrT58* Δ*galU183* Δ*trpE5 minB2 rfb-2 recA56 relA1* Δ*thyA57 endA1 oms-1* Δ*asd-4 rpoB402 cycB2 cycA1 hsdR2*	8
χ2338	F⁻ Δ*araC766 fhuA53 dapD8 minA1 gltA16* Δ69(*gal-chlD*) λ⁻ Δ*trpBC13 minB2 rfb-2 gyrA25* Δ*thyA57 endA1 oms-1 aroB15 cycB2 cycA1 hsdR2*	17 steps from χ1776 (15)
χ2341	F⁻ Δ*araC766 fhuA53 dapD8 proA318 minA1* Δ69(*gal-chlD*) λ⁻ Δ*trpBC13 minB2 rfb-2 gyrA25* Δ*thyA57 endA1 oms-1* Δ*asd-4 cycB2 cycA1 hsdR2*	8
χ2367	χ2341 lysogenized with λ *c*I857 *b2 red*β3 *S7*	This study
χ2382	HfrOR91 *thr-16 tsx-63 purE41 glnV42* λ⁻ *trpE63 his-53 recA56 relA1* Δ*thyA57 tte-1 mtlA9 metE98 cycB2 cycA1* (O-*lysA argA his...serA* F)	Isolated in 14 steps from χ540 (14)
χ2763	RR1 lysogenized with λ *c*I857 *b2 red*β3 *S7*	This study
χ2764	HB101 lysogenized with λ *c*I857 *b2 red*β3 *S7*	This study
χ2811	F⁻ *lacY1 glnV44 galK2 galT22* λ⁻ Δ*thyA57 metB1 hsdR2*	χ2382 × WA802
χ2813	F⁻ *lacY1 glnV44 galK2 galT22* λ⁻ *recA56* Δ*thyA57 metB1 hsdR2*	χ2382 × WA802
χ2815	χ2811 lysogenized with λ *c*I857 *b2 red*β3 *S7*	This study
χ2819	χ2813 lysogenized with λ *c*I857 *b2 red*β3 *S7*	This study
χ2831	χ2813 lysogenized with λ *c*I857 *b2 red*β3	This study
χ2844	F⁻ *tsx-462*::Tn*10* λ⁻	Selected as a T6ʳ Tcʳ transductant of MG1655 (26) using λ561 (35)
χ2845	F⁻ *ara-14 leu-6 azi-6 fhuA29 lacY1 tsx-462*::Tn*10 lon-9 purE42 glnV44 galK2* λ⁻ *trpE38 lpcB rpsL73*	P1L4 (χ2844) → RCG103 (23)
χ2865	F⁻ *lacY1 glnV44 galK2 galT22* λ⁻ *metB1 malE*::Tn*10 hsdR2*	P1L4 (TST1) → WA802 (TST1 from T. Silhavy)
χ2866	F⁻ *lacY1 glnV44 galK2 galT22* λ⁻ *metB1 uvrA1 hsdR2*	P1L4 (χ2660) → χ2865, selected as a Mal⁺ UVˢ transductant
χ2867	F⁻ *lacY1 tsx462*::Tn*10 lon-9 glnV44 galK2 galT22* λ⁻ *metB1 uvrA1 hsdR2*	P1L4 (χ2845) → χ2866
χ2875	F⁻ *lacY1 tsx-465 lon-9 glnV44 galK2 galT22* λ⁻ *metB1 uvrA1 hsdR2*	From χ2867[b]

[a] LE392 is a calcium-resistant isolate of strain ED8654 (44) that was isolated by Lynn Enquist (personal communication).
[b] Selected as a fusaric acid-resistant derivative (39).

methylene blue O (EMBO) agar (24) 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 (12) and 10 mM Tris (pH 7.4)–10 mM $MgSO_4$–0.1% gelatin–100 mM NaCl (TMGS) were used as diluents for bacteria and lambdoid particles, respectively.

Construction of lysogens. Bacteriophage λ 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 supplemented with methionine, THD, and 20 μg of trimethoprim (Sigma Chemical Co., St. Louis, Mo.) per ml. Isolates which required THD for growth, which is indicative of the presence of the *thyA* mutation, were subsequently tested for the phenotypic properties of *recA* and *relA* mutations by sensitivity to UV light (7) and the inability to grow on minimal glucose plates supplemented with THD at 40 μg/ml and with serine, methionine, and glycine at 100 μg/ml (52), respectively. Selection for rare λ *b2* lysogens involved the plating of cells to which the λ *b2* phage had been preadsorbed for 30 min, along with 10^9 λ *b2 c* and 10^9 λ *h*φ80 Δ*att c* on EMBO agar. Pseudolysogens gave rise to irregularly shaped purple colonies as a result of phage infection of λ-sensitive cells that occurred during growth, while true lysogens appeared as perfectly round, white, nonmucoid colonies (24) 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 were excised at a very low frequency, and this did not result in lysis of the indicator LE392 cells, whereas tandem polylysogens excised and lysed the indicator.

Enzymes. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, Inc., Boston, Mass., or Bethesda Research Laboratories, Gaithersburg, Md. DNase I 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 by the technique described by 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 Maniatis et al. (40). To prevent ligation of tandem pMMB34 vectors, the right and left arms were prepared in a manner analogous to that described by Ish-Horowicz and Burke for pJB8 (32). pMMB34 was totally digested with either *Hpa*I or *Hin*dIII, treated with alkaline phosphatase, extracted with phenol-chloroform, and digested with *Bam*HI; 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 described previously (9, 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 described previously (42). *M. vaccae* DNA was partially digested with either *Bam*HI or *Sal*I 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 packaged in vitro. The in vitro-packaged *Bam*HI- or *Sal*I-generated libraries were transduced into χ2367 and χ2764, respectively; and recombinant clones were selected on L agar containing 25 μg of ampicillin per ml.

S. typhimurium DNA was partially digested with *Sau*3A, and the DNA fragments were separated by size by centrifugation, as described previously (9). Fragments with an average size of 35 kb were mixed with a fourfold molar excess of each of the pMMB34 arms, ligated, and packaged in vitro. The resulting particles were transduced into χ2764, and recombinant clones were selected on L agar containing 50 μg of kanamycin per ml.

Samples of *S. mutans* DNA, partially digested with *Sau*3A, *Eco*RI, or *Pst*I, were also separated by size by sucrose gradient centrifugation. A library of DNA sequences contiguous within the *S. mutans* chromosome was constructed by ligating DNA fragments greater than 40 kb to *Pst*I-cut pHC79. Libraries of DNA fragments that were not contiguous within the *S. mutans* chromosome were generated in two ways. *Eco*RI-cleaved DNA fragments of 5 to 10 kb were ligated 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 was size fractionated as described above. DNA fragments of greater than 40 kb 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 4:1 molar ratio of vector to insert. Again, the ligated DNA fragments were packaged in vitro, and the resulting particles were transduced into χ2819. Recombinant clones were selected on L agar plates containing either 10 μg of tetracycline per ml for the *Pst*I and *Eco*RI libraries of 25 μg of ampicillin per ml for the *Sau*3A library. All plates used for selection were incubated 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 at the original culture volume. Transduction with 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 during a 30-min incubation period at 30°C. After adsorption, 0.7 ml of superbroth was added to the transducing particle-cell suspension, which was then incubated for an additional 45 min at 30°C to allow expression of antibiotic resistance genes. Recombinant clones were selected for growth at 30°C on L agar or in L broth containing 25 μg of ampicillin per ml, 10 μg of tetracycline per ml, or 50 μg of kanamycin per ml. Individual recombinant clones or libraries were amplified by growing the clones or pools of clones in L broth containing 0.4% glucose and the appropriate 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 2-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 at 30°C with moderate shaking to an optical density of 0.3. The prophage were induced by incubating the flasks for 15 min in a water bath at 45°C with occasional shaking, followed by incubation with vigorous shaking at 37°C for 1 to 5 h. After incubation at 37°C, the cells were lysed by adding a 0.01 volume of chloroform and shaking for 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 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. The yields of repackaged recombinant cosmids, assayed at 1-h intervals for 5 h after induction, did not significantly increase above 10^9 repackaged recombinant cosmids per ml of lysate after 2 h of induction (data not shown).

High-titer lysates of in vivo-packaged recombinant cosmids were prepared by incubating the 2-liter flasks with vigorous aeration for 2.5 h at 37°C after the initial induction. The induced, unlysed cells were pelleted by centrifugation, suspended in 7.0 ml of TMGS, and lysed with 1% chloroform. This suspension was treated with DNase I (100 μg/ml), and the cell debris was pelleted by centrifugation. The packaged recombinant phage could be further purified by cesium chloride equilibrium density gradient centrifugation. Yields of 2.5×10^{11} to 5×10^{11} transducing particles conferring antibiotic resistance were routinely obtained from 500-ml cultures.

Complementation analysis. Repackaged recombinant cosmid molecules were transduced into cells of the particular test strain, prepared as described above for λ infection, 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 of superbroth was added, and the cells were incubated for an additional 45 min to allow expression of the transduced drug resistance genes. The cells were then diluted and spread on selective media.

Expression analysis. The protocol for expression analysis 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 a mutation in the *lon* gene was introduced into χ2875 to determine its effect on the stability of expressed foreign proteins. Cells (20 ml) were grown to 1×10^8 cells per ml in minimal salts broth containing 0.4% maltose and all 20 amino acids at the previously described concentrations (13). The cells were pelleted at 8,000 rpm in an Sorvall SS34 rotor for 10 min at room temperature. The pellet was suspended in 100 ml of minimal salts broth without supplements and irradiated in a sterile flat-bottomed porcelain evaporating dish (diameter, 6 in.) with 3,000 J/m^2 of UV light. The cells were pelleted as described above and suspended in a 1/10 volume of growth medium containing 10 mM $MgSO_4$ but without methionine. Cells (1 ml) were transferred to an Eppendorf tube and incubated at 37°C for 2 h to allow degradation of existing mRNA. Ten repackaged recombinant phage were added per cell and allowed to adsorb for 15 min prior to the addition of 10 μCi of [^{35}S]methionine (1,000 Ci/mol). After 10 min of labeling, cells were lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9).

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. After 45 min of incubation for the expression of the antibiotic resistance gene, the transduction mixture was diluted and spread to yield 200 to 300 antibiotic-resistant colonies per selective plate. The plates were incubated at 30°C for 18 to 20 h so that the average colony size was 0.75 mm. To screen for dextranase activity, recombinant clones were plated on L agar supplemented with THD and ampicillin or tetracycline. Colonies were gently overlayed with 12 to

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

Recombinant cosmid	No. (10^8) of repackaged recombinant cosmids per ml of lysate[b]	Yield (no.) of repackaged recombinant cosmids per Ap^r cell
pYA1010	2.1	5
pYA1011	3.5	2
pYA1012	3.8	10
pYA1013	1.2	9

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

[b] Titers were determined as the number of transducing particles conferring Ap^r to HB101 cells.

14 ml of sterile 1.4% agar (55°C) containing 0.05 M sodium acetate (pH 5.4) and 0.7% blue dextran. To lyse cells in colonies by thermal induction of the λ prophage, plates were incubated in an inverted position for 8 h at 42°C and then for an additional 24 h at 37°C. The production of dextranase by a recombinant clone resulted in a zone of clearing in the blue dextran agar overlay. Protease activity was detected in a similar fashion by overlaying colonies with 12 to 14 ml of sterile 1.4% agar (55°C) containing 0.05 M sodium phosphate buffer (pH 6.8) and 5% skim milk followed by thermally induced lysis. 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 T10) that are needed to detect enzyme activity (a modification of a technique developed by R. Russell, personal communication). Recombinant clones were plated on L agar supplemented with THD, ampicillin or tetracycline–0.4% sucrose–0.2% dextran T10. After sufficient growth, the colonies were incubated at 42°C for 8 h followed by an additional 48 to 72 h of incubation at 25°C. A large, white, domelike and mucoidlike appearance was considered positive for glucosyltransferase activity. In all three cases, transducing particles conferring ampicillin resistance (Ap^r) or tetracycline resistance (Tc^r) and the observed activities were recoverable from the screened colonies.

RESULTS

Amplification of packaged recombinant cosmid molecules. 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-titer lysates prepared by inducing individual clones of χ2367 containing pHC79::*M. vaccae* molecules transferred Ap^r 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^r. When the λ-resistant strain χ1849 (27) was used as a recipient host, transfer of Ap^r was 3 to 4 logs less than transfer to λ-sensitive cells (data not shown). A similar transfer of Tc^r was observed for several individual pHC79::*M. leprae* recombinant molecules derived from a previously described cosmid library (9) when packaged in vivo in χ2819. In all cases, restriction endonuclease analysis of plasmids isolated from in vivo-packaging strains containing recombinant clones prior to in vivo packaging and from a pool of thousands of cells obtained after transduction with the respective lysate showed identical DNA banding patterns. A representative restriction digestion analysis is shown in Fig. 1. We thus conclude that transfer of antibiotic resistance is mediated by the in vivo-packaged recombinant cosmids and that in vivo packaging does not alter 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 a possibility, the recombinant cosmid pYA1010 was transduced into the isogenic *recA*$^+$ χ2763 and *recA13* χ2764 strains and repackaged in these strains. The yields of transducing particles conferring Ap^r were 8- to 10-fold greater in χ2763 or χ2764 compared with that in χ2367. However, the absence of the wild-type *recA* gene product did not significantly affect the yields of repackaged recombinant cosmid molecule pYA1010 (Table 3). Restriction analyses of pYA1010 isolated as a plasmid before in vivo packaging and again after transduction showed identical banding patterns when either the *recA* or Rec$^+$ isogenic packaging strain was used. The λ *c*I857 *b*2 *red*β3 *S*7 prophage in these strains excises at very low frequencies and can be assayed by plating on a strain containing a *tyrT* (*supF*) mutation. There was no significant difference between the yields of PFUs from these lysates as a result of the *recA* mutation (Table 3).

Complementation analyses. A contiguous genomic library of *S. typhimurium* DNA was constructed in pMMB34. A total of 928 kanamycin-resistant (Km^r) clones were screened

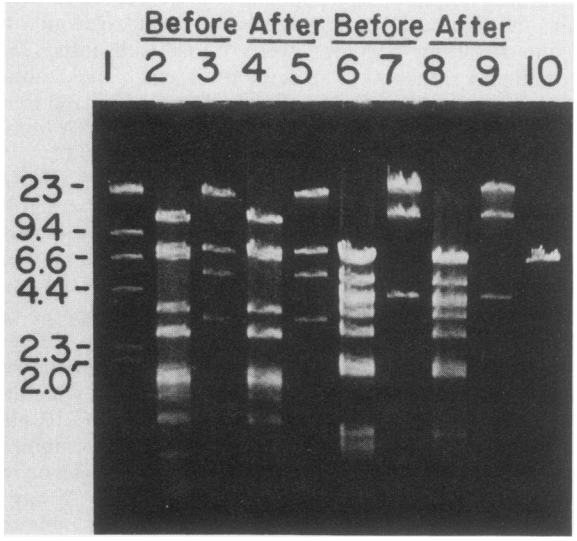

FIG. 1. Agarose gel electrophoretic analysis of restriction digests of pYA1061 and pYA1062 plasmid DNA isolated from before in vivo packaging in χ2819 and after transduction of in vivo-packaged cosmids into χ2866. Lane 1, λ DNA digested with *Hin*dIII (sizes are given in kilobases to the left of the gel); lanes 2 and 3, pYA1061 isolated before in vivo packaging and digested with *Pst*I and *Hin*dIII, respectively; lanes 4 and 5, pYA1061 isolated from χ2866, after transduction and selection for Tc^r, digested with *Pst*I and *Hin*dIII, respectively; lanes 6 and 7, pYA1062 isolated before in vivo packaging and after digestion with *Pst*I and *Hin*dIII, respectively; lanes 8 and 9, pYA1062 isolated from χ2866, after transduction and selection for Tc^r, digested with *Pst*I and *Hin*dIII, respectively; lane 10, pHC79 digested with *Pst*I.

for the presence of recombinant molecules that complemented mutations in the *ara*, *leu*, *pro*, and *gal* loci of the host. Based on average insert sizes of 37 kb and assuming that there was no biasing against the cloning of any fragment of DNA, the library comprised 99.98% of the *S. typhimurium* genome (10). Three to nine recombinant clones were found in the original library that complemented each of the four mutations (Table 4). All three of the recombinant cosmids that complemented the *ara* mutation also complemented the *leu* mutation, which is consistent with their close proximity of less than 0.5 min on the *S. typhimurium* genetic linkage map (47).

An amplified library of repackaged pMMB34::*S. typhimurium* DNA molecules was prepared from a pool of clones and was screened for the number of recombinant cosmids that complemented the *ara*, *leu*, *pro*, *gal*, and *xyl* mutations in strain 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 4). However, even with this drop, 10^4 recombinant cosmids that complemented the *ara* mutation were present per ml of amplified lysate.

A genomic library of pHC79::*M. vaccae* DNA recombinant molecules was constructed and amplified in χ2764. None of the 370 Ap^r Tc^s clones, representing 96% of the *M. vaccae* genome (9, 10), contained DNA sequences which complemented the *ara*, *leu*, *pro*, and *gal* mutations of χ2764 or the *pro*, *thyA*, *trpE*, *ara*, *gal*, or *lac* mutations of χ2001. However, restriction endonuclease analyses of 10 random Ap^r transductants of χ2001 revealed a variety of cloned sequences present, indicating that the library was diverse.

Further characterization of the pHC79::*M. leprae* libraries described previously (9) was done by transducing χ2338 and screening for complementation of the *dapD*, *gltA*, Δ*trpBC*, Δ*thyA57*, 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 using the isogenic *uvrA* χ2866 and *uvrA lon* χ2875 strains. The *lon* gene encodes a protease that is known to degrade abnormal proteins of *E. coli* (5, 25, 48), as well as fusions of foreign proteins to β-galactosidase (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 when recombinant libraries are screened.

High-titer lysates of three individual repackaged recombinant pHC79::*M. leprae* molecules were prepared for infection into UV-irradiated χ2866 and 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 10, followed by labeling with [^{35}S]methionine. The cells were lysed, and polypeptides were run on a sodium dodecyl sulfate-polyacrylamide gel. A few polypeptides other than the Tc^r gene product were synthesized at low levels from two of the three recombinant molecules analyzed. The intensity of the band of one of the newly synthesized polypeptides was greater in the strain containing the *lon-9* mutation compared with 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 translation 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 three different recombinant cosmid libraries: one contiguous and two noncontiguous gene libraries. The libraries were amplified in χ2819 by in vivo cosmid packaging and subsequently transduced into χ2831, which contains a prophage that possesses a normal *S* gene, thereby permitting cell lysis to facilitate in situ screening (Fig. 2). Dextranase, glucosyltransferase, and protease activities were found among the recombinants of the different libraries (Table 5). Dextranase-positive clones were found in all three libraries, with two distinguishable phenotypic types being found in the *Sau*3A library. One type gave a much more pronounced zone of clearing on blue dextran agar compared with the other type, which was found in all three libraries. The glucosyltransferase and protease activities were only found in the two noncontiguous libraries.

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

Recombinant cosmid clone	Yield (no.) of repackaged recombinant cosmids per Ap^r cell[a]	PFUs (10^{-4}) per repackaged recombinant cosmid[b]
χ2763(pYA1010) *recA*$^+$	51	3.6
χ2764(pYA1010) *recA*	44	1.0

[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. Titers of repackaged recombinant cosmids were determined as the number of transducing particles conferring Ap^r to HB101 cells.

[b] Titers of PFUs were determined on LE392 cells.

TABLE 4. 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	Frequencies of complementing recombinant cosmids in:	
	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[c]	1.5×10^{-3}

[a] Frequencies in the original in vitro-packaged library of 928 recombinant cosmids conferring Km^r.

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

[c] ND, Not determined.

DISCUSSION

We constructed a set of thermoinducible, excision-defective lambda lysogens of *E. coli* K-12 that permitted the repackaging of recombinant cosmid molecules into bacteriophage lambda-transducing particles in vivo. Thermal induction of these strains containing recombinant cosmid molecules as plasmids yielded high titers of repackaged recombinant cosmid molecules. These strains were all lysogenized with a λ prophage, the genome of which could be excised efficiently from the *E. coli* chromosome. Induc-

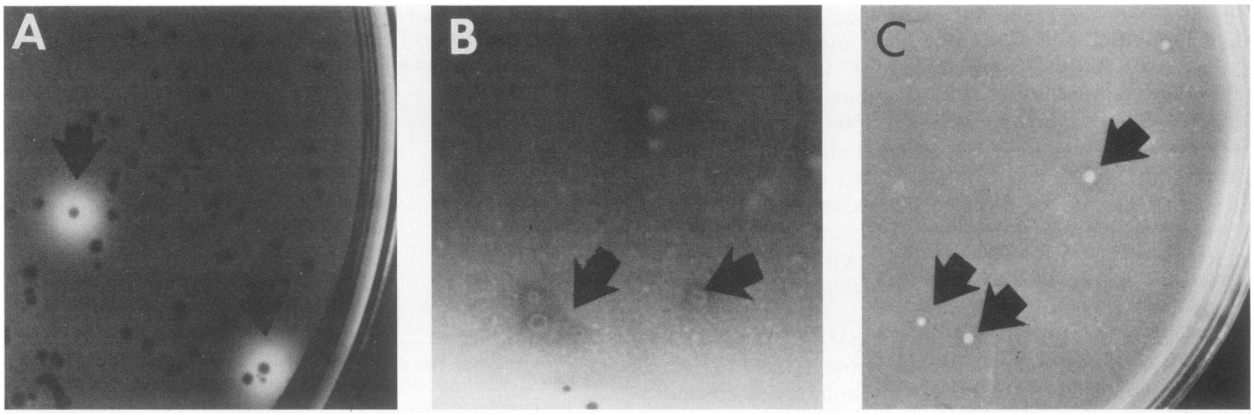

FIG. 2. In situ screening assays for dextranase (A), protease (B), and glucosyltransferase (C) activities. χ2831 was transduced with various *S. mutans* cosmid libraries and transductants selected on antibiotic-selective media. See the text for the screening assays to detect clones expressing dextranase, protease, and glucosyltransferase.

tion of these strains containing recombinant cosmid molecules resulted in a lysate that contained only one plaque-forming phage for every 10^4 to 10^5 repackaged recombinant cosmid molecules. This repackaging of recombinant cosmid molecules did not depend on recombination systems of the host or phage, which is consistent with previous findings (19, 22, 37, 41). Lysogens that package in vivo have been made and tested in three different genetic backgrounds. χ2367 was constructed as an EK2 host but yielded about a log fewer repackaged recombinant cosmids than the HB101 and WA802 derivatives. The WA802 derivatives had an advantage over the HB101 derivatives since they contained an *hsdR* mutation rather than an *hsdS* mutation. Both hosts were defective in host restriction activity, but the WA802 derivatives were proficient in host modification activity, thereby allowing modification of the cloned DNA. This facilitated complementation analysis and other experiments designed to use the extensive collection of available *E. coli* K-12 strains that are mostly restriction proficient. Isogenic strains that package in vivo which differ in production of the prophage *S* gene-specified endolysin offer versatility. χ2819, which contains a defective *S* gene, is an excellent strain for preparation of high-titer lysates of in vivo-packaged recombinant cosmids since the induced, unlysed cells containing in vivo-packaged recombinant cosmids could be concentrated by centrifugation prior to lysis with chloroform. χ2831, which contains a normal *S* gene, facilitated screening of expressed clone DNA, since cells in colonies were easily lysed after thermoinduction to permit in situ screening of products present within the cell.

Construction of genomic libraries provides genetic material for analysis. Amplified cosmid libraries can also be an alternative source of genomic DNA from organisms which are difficult to grow (e.g., *M. leprae*) or the DNA of which is difficult to extract or both. In either case, it is important that the library represent the entire genome. The amplification of any library of DNA sequences will 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 it 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 rather than as 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 some of these can be circumvented. The presence of two tandem vector molecules within a recombinant molecule causes instability because recombination yields a vector 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 a way that precludes 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, e.g., instability as a result of the inability to be replicated or instability due to sequences that lead to recombination to delete a sequence. The ability to package in vivo in the absence of host and phage recombination systems minimizes the loss of sequences due to homologous but not to nonhomologous recombination.

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 cells containing such sequences will increase within the population. The precise nature of the instability of the *ara*-complementing clone of the *S. typhimurium* DNA library is unknown. The library was prepared in such a way as to preclude tandem vector formation. Based on the

TABLE 5. Screening of *S. mutans* cosmid libraries for dextranase, protease, and glucosyltransferase activities

Library	No. of original clones	Library description	No. of positive clones/total no. screened for:		
			Dextranase	Glucosyl-transferase	Protease
*Pst*I	1,200	Sized, contiguous	6/2,100	0/4,000	0/4,000
*Eco*RI	1,700	Sized, noncontiguous	3/4,200	3/2,400	0/4,300
*Sau*3A	$>10^4$	Unsized	13/2,900	4/1,200	3/4,800

suppositions presented above for DNA that is known to be expressed in *E. coli*, certain recombinant molecules within the *S. typhimurium* library should decrease in the population, while others should increase.

We constructed contiguous and noncontiguous libraries with *S. mutans* DNA, assuming that when such large segments of DNA were cloned it was possible that genes of interest could be physically linked to genes the presence of which might be deleterious to the growth of the host. The inability to obtain an *S. typhimurium* recombinant cosmid that only complemented the *ara* mutation and not the *leu* mutation suggests that sequences near *ara* but distal from *leu* might confer instability or lethality. The construction of noncontiguous libraries would physically dissociate existing linkages. Recombinant cosmids specifying glucosyltransferase and protease activities were only found in the noncontiguous gene libraries. Phenotypes which require the interaction of two or more unlinked genes widely separated on a chromosome cannot be found in contiguous libraries but should be found at frequencies of 10^{-4} or less in a noncontiguous gene library. The possibilities that the glucosyltransferase and protease activities require one or more gene products or that the genes encoding these activities are linked to genes that are deleterious to *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. 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 because they are restriction proficient. Use of *E. coli* host strains possessing an *hsdR* mutation allows introduction of foreign DNA without restriction and modifies DNA so that amplified cosmid recombinants can be introduced into any restriction-proficient *E. coli* host for complementation analysis.

Preparation of high-titer lysates of in vivo-packaged recombinant cosmid molecules, with low numbers of background helper phage, permits analysis of the polypeptides produced by a particular recombinant molecule following infection of UV-irradiated cells. The maxicell technique of expression analysis is dependent on the statistical probability that a plasmid is less likely to incur DNA damage than the chromosomal DNA (46). Molecules that do incur 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. Transduction of UV-irradiated cells with recombinant cosmids is therefore a useful alternative for expression analysis. The use of this technique for analysis of *M. leprae* recombinant cosmids provided an efficient means of screening large segments of *M. leprae* DNA for their ability to be expressed in *E. coli*. The results obtained, however, revealed that only a small fraction of the potential *M. leprae* genes are expressed. The use of χ2866 and χ2875, which contain *lon*$^+$ and *lon-9* alleles, respectively, for analysis of expression of recombinant cosmid DNA offers a means of testing the effect of the *lon* protease on the stability of foreign proteins.

Another use of strains that package in vivo for analyses of cosmid libraries was demonstrated by 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 replicating the screened library. This method is not only applicable to enzyme screening but to immunological screening 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. (54) have demonstrated that a lysate made on induction of a λ *c*I857 lysogen containing a recombinant cosmid molecule and a transposon inserted in the bacterial chromosome results in the packaging of recombinant cosmid molecules into which the transposon had inserted. Transposon-induced mutations in the recombinant cosmid molecule could be identified by transducing a suitable host and selecting for growth on plates containing antibiotics for which the transposon and the cosmid conferred resistance. However, the high titers of infectious phage in their lysates hindered the effectiveness of this technique. The use of strains that package in vivo with nonexcisible, thermoinducible λ prophages should eliminate this problem.

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

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