1979

Construction Of Safer Escherichia Coli For Use In Recombinant Dna Experimentation.

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The University of Alabama in Birmingham PH.D. 1979

CONSTRUCTION OF SAFER *ESCHERICHIA COLI* FOR USE IN RECOMBINANT DNA EXPERIMENTATION

by

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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 in Birmingham

BIRMINGHAM, ALABAMA

1979

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Dennis Anthony Pereira

Major Subject Microbiology

Title of Dissertation Construction of Safer Escherichia coli for Use in Recombinant DNA Experimentation

Dissertation Committee:

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Director of Graduate Program _____

Dean, UAB Graduate School _____

Date 8/17/79

ACKNOWLEDGEMENTS

I acknowledge the helpful discussions and suggestions given by the Curtiss laboratory group, especially those individuals who participated in the various strain construction projects. I thank Anthony Maurelli for finishing an experiment in my absence and offer special thanks to Raúl Goldschmidt for his continued controlling influence and helpful daily discussions. Roy Curtiss III deserves and is offered my very special thanks for his patience, teaching and friendship during an eventful graduate career. Finally, my wife and daughter deserve my sincerest appreciation for their sacrifices, both financial and emotional, in allowing me to continue toward this goal.

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA IN BIRMINGHAM

Degree Ph.D. Major Subject Microbiology

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Title Construction of Safer Escherichia coli for Use in Recombinant DNA Experimentation

Derivatives of Escherichia coli K-12 were constructed which are useful hosts for recombinant DNA research in which virulent lambda (λ) is used as a vector. These strains, χ1953 and χ2447 possess additional genetic lesions which increase their biological containment properties. The genotype of χ1953 is F^- tonA53 dapD8 lacY1 supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 hsdS3 and χ2447 is F^- dapD22 ΔlacZ39 Δ47[gal-uvrB] tyrT58 nalA29 ΔthyA57 endA1 asd-3 hsdS3.

Prior to this report χ1953 was certified as a component of an EK2 host-vector system. χ2447 is proposed as an improved host for virulent λ vectors with both increased utility and safety properties over that of χ1953. Further, a sibling of χ2447 was constructed, χ2281, which because of its Su^0 phenotype makes it a highly contained and convenient testing strain in which to test the safety properties of chimeric λ vectors.

Phenomena which affected the kinetics and extent at which Dap^- bacteria underwent diaminopimelic acid-less (DAP-less) death were also investigated. It was shown that Dap^- bacteria survived DAP-less death by secreting an exopolysaccharide slime provided appropriate concentrations of certain monovalent and divalent cations were present. A

Δ[gal-uvrB] isogenic sib which did not possess the ability to synthesize an exopolysaccharide slime was not able to survive a similar DAP-starvation. The exopolysaccharide proficient sib was shown to lose the ability to survive DAP-less conditions if the temperature and/or salts present were adjusted to inhibit exopolysaccharide production.

The kinetics and regrowth of Dap^- bacteria deprived of DAP was studied. Dap^- bacteria were found to possess the ability to survive and even regrow in media lacking DAP. The survivors of the DAP-less incubation remained Dap^-. The regrowth was found to be dependent on the presence of salts in the media.

Abstract Approved by: Committee Chairman

Program Director

Date 8/17/79 Dean of Graduate School

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LIST OF SYMBOLS AND ABBREVIATIONS

The following symbols and abbreviations are used in this dissertation.

Superscript + = wild type gene and/or phenotype

Superscript - = mutant gene and/or phenotype

Superscript R = resistant

Superscript S = sensitive

(am) = gene preceding possesses amber mutation

Amp.-Cyc. = ampicillin-cycloserine

Bio = biotin

BSG = buffered saline gelatin

Ch3A = Charon 3A

Ch4A = Charon 4A

Ch16A = Charon 16A

Ch21A = Charon 21A

DAP = diaminopimelic acid

E. coli = *Escherichia coli*

EK1 = biological containment level one for *E. coli* K-12

EK2 = biological containment level two for *E. coli* K-12

EK3 = biological containment level three for *E. coli* K-12

EMB = eosin methylene blue

Fuc = fucose

Gal = galactose

Glc = glucose

GlcA = glucuronate

IPP = C-55-isoprenoid lipid carrier

Lac = lactose

LPS = Lipopolysaccharide

MA = minimal salts agar

MA-CAA = minimal salts agar supplemented with Casamino acids and Glc

Met = methionine

ML = minimal salt liquid medium

ML-CAA = minimal salt liquid medium supplemented with Casamino acids and Glc

PA = penassay agar

pfu = plaque forming units

P1 = physical containment level one

P2 = physical containment level two

P3 = physical containment level three

P4 = physical containment level four

r^-m^- = restriction deficient, modification deficient

rpm = revolutions per minute

sel'n. = selection

Su^0 = phenotypic designation indicating lack of suppressor + RNA

SuII = phenotypic designation of supE gene

SuIII = phenotypic designation of tyrT gene

Thd = thymidine

Thr = threonine

$t_{½}$ = half-life

UDP = uridine diphosphate

UV = ultraviolet irradiation

v/v = concentration adjusted on volume to volume basis

$\underline{\lambda}$ = bacteriophage $\underline{\lambda}$

CHAPTER 1

INTRODUCTION

The National Institutes of Health (NIH) have adopted guidelines to ensure that the practice of recombinant DNA research does not cause harm to the public environment (25). This objective is achieved by stipulating that experiments be conducted using various levels of physical and biological containment. There are four levels of physical containment ranging from the lowest, P1, which simply mandates proper microbiological practices to the highest, P4, which requires elaborately constructed laboratories. Whatever the level of physical containment, the rationale is to prevent the inadvertent escape of the progeny of recombinant DNA work. Biological containment involves the use of a host bacterium and vector (plasmid or virus) which have been intentionally modified to reduce the probability of an escaped chimera becoming established in the biosphere. The NIH guidelines describe three levels of biological containment (EK1, EK2, and EK3) which define decreasing probabilities of a bacterium's ability to survive and transmit its vector under a series of defined experimental conditions. Requirements for EK1 are substantially a property of the containment that is inherent in Escherichia coli K-12 strains (10). EK2 and EK3 certification requires that the probability of perpetuating a cloned DNA fragment under non-permissive conditions, designed to represent the natural environment, be $\leq 1 \times 10^{-8}$ (1).

The first permanently approved EK2 host-vector system (χ1776 in conjunction with pSC101) was constructed by Curtiss et al (13) and χ1776 in conjunction with the plasmid vectors, pMB9, pBR322 and pBR313 has since been certified as EK2 (25). Shortly after χ1776's certification several investigators applied for and received EK2 approval for a total of seven lambda (λ)-host systems (6,15,18; F. Blattner, personal communication). Only the system developed by Donoghue and Sharp qualified for EK2 certification independent of a disabled propagating host (15). The most commonly employed phage vectors are those developed by Blattner et al (6); Charon 3A (Ch3A), Charon 4A (Ch4A), Charon 16A (Ch16A), and Charon 21A (Ch21A) and by Leder et al (18); λgtWES·λB and λgtWES·λB' (18). The latter vectors were not able to satisfy the NIH criteria for EK2 certification because of their propensity to perpetuate model cloned fragments. To circumvent this problem the approved EK2 host, χ1953, also known as DP50, was constructed to provide the additional safety margin needed for EK2 certification. The term "EK2 host" refers to the bacterial component of a EK2 host-vector system and "EK2 vector" to the viral component. Leder et al introduced the amber suppressor tyrT58 into χ1953 (now called χ2098 or DP50 supF) to provide a suitable propagating host for λgtWES·λB and λgtWES·λB'.

The construction and properties of χ1953 as well as a newly developed host for virulent λ vectors are described in this paper. The goal was to develop a system which would be certifiable as EK3. Ch4A was selected for the proposed EK3 host-vector system because

of its intrinsic safety and because of its large cloning capacity (6). Care was taken, in the construction of the hosts (χ1953 and χ2447), to incorporate mutations which; i) allow the propagation of all currently certified vectors, ii) maximize the biological containment of the host and, iii) facilitate the testing of the above vectors. The χ2447-Ch4A system, reported here, is unique in two respects. The system possesses a high level of biological containment due to the independent safety properties of the vector, Ch4A, as well as those intrinsic to the propagating host, χ2447. The novel feature of the χ2447-Ch4A system is the development of an equally contained strain, χ2281, that allows for the convenient testing of the safety properties of the phage containing cloned DNA fragments.

MATERIALS AND METHODS

Bacterial and Bacteriophage Strains. The bacterial strains used are described in Table 1. The gene symbols are those suggested by Bachmann et al (3) and allele numbers when listed have been assigned by the Coli Genetic Stock Center. Bacteriophages T5, λvir, λcI857, λNNcI857, λcI857, J227, 434, P1kc, P1clm clr100 and P1L4 were all obtained from the laboratory of Roy Curtiss. λNNcI857·h434 was constructed for the isolation of supE$^+$ derivatives. Ch4A was kindly provided by F. Blattner.

Media. The complex media used were: Penassay agar (PA; Difco), λ broth [10 g tryptone (Difco), 5 g yeast extract (Difco) and 5 g NaCl/l and 10 mM $MgSO^4$] superbroth (21), L broth (19), EMB Difco; EMB Agar Base containing 5 g NaCl and 5 g yeast extract/l) and MacConkey Base Agar (Difco). Broth media for λ and P1 were solidified with 12 g and 6 g agar (Difco)/l for bottom and top soft agars, respectively. Media for P1 was supplemented to 25 mM $CaCl_2$. Sterilized carbon sources (added to 1% v/v), antibiotics and bile salts (Difco Bile Salts No. 3) were added after autoclaving to the concentration listed in the text.

The synthetic media were ML and MA (9) and were supplemented with sterile carbon sources (0.5%), amino acids, purines, pyrimidines and vitamins after autoclaving (12). Casamino acids media contained

TABLE 1. Bacterial strains[a]

Strain	Mating Type	Genotype
χ289	F⁻	supE42
χ341	Hfr Cav	proC relA1 metB1
χ420	F⁻	thr-1 ara leu-6 pan tonA-21 proB lacY-1 tsx purE supE44 rpsL thi-1 pdx arg
χ559	Hfr OR11	leu-45 supE42 ΔthyA57
χ573	F' ORF-4	Δ71[lac-purE] serA12
χ1038	F⁻	lacY1 supE44 galK2 galT22 metB1 hsdS3
χ1633	F⁻	thr16 tsx63 purE41 supE42 Δ[trpBC] his53 xyl4 cycB2 cycA1
χ1702	F⁻	tonA53 dapD8 minA1 purE41 supE42 minB2 his53 metC65 ilv-277 cycB2 cycA1 hsdR2
χ1753	F⁻	tsx63 supE42 his-53 lysA32 xyl-14 arg-65
χ1821	Hfr OR11	supE42 endA1
χ1918	Hfr H	lacZ argE relA1 metB1 rpsL
χ1947	F⁻	lacY1 supE44 metB1 hsdS3
χ1948	F⁻	lacY1 supE44 Δ47[gal-uvrB] metB1 hsdS3
χ1949	F⁻	lacY1 supE44 Δ47[gal-uvrB] nalA29 metB1 hsdS3
χ1950	F⁻	lacY1 supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 metB1 hsdS3
χ1951	F⁻	lacY1 supE44 nalA29 ΔthyA57 metB1 hsdS3
χ1952	F⁻	lacY1 supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 hsdS3

TABLE 1. (Continued)

Strain	Mating Type	Genotype
χ1953	F⁻	tonA53 dapD8 lacY1 supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 hsdS3
χ1954	F⁻	tonA53 dapD8 lacY1 supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 serA12 hsdS3
χ1955	F⁻	tonA53 dapD8 lacY1 supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 endA1 hsdS3
χ2013	F⁺	tyrT58 mel
χ2017	F'	ΔlacZ39 trp
χ2087	F⁻	leu-50 Δ69[lacZOPI] tsx-98 supE42 Δ40[gal-uvrB] rpsL206 argH70
χ2100	F⁻	lacY1 galK2 galT22 nalA metB1 hsdS3
χ2170	F⁻	thr ara leu dapD proB lacY tsx purE rpsL thi pdx arg
χ2171	F⁻	leu-50 Δ69[lacZOPI] tsx-98 supE42 Δ40[gal-uvrB] asd-3 rpsL206 argH70
χ2191	F⁻	tonA52 dapD8 lacY1 supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 serA endA1 hsdS3
χ2192	F⁻	proC supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 serA endA1 hsdS3
χ2193	F⁻	ΔlacZ39 supE44 Δ47[gal-uvrB] nalA29 ΔthyA57 serA endA1 hsdS3
χ2194	F⁻	ΔlacZ39 supE44 Δ47[gal-uvrB] Δ[trpBC] nalA29 ΔthyA57 serA endA1 hsdS3
χ2195	F⁻	tonA53 dapD8 ΔlacZ39 supE44 Δ47[gal-uvrB] Δ[trpBC] nalA29 ΔthyA57 serA endA1 hsdS3
χ2196	F⁻	pan tonA ΔlacZ39 supE44 Δ47[gal-uvrB] Δ[trpBC] nalA29 ΔthyA57 serA endA1 hsdS3
χ2197	F⁻	dapD22 ΔlacZ39 supE44 Δ47[gal-uvrB] Δ[trpBC] nalA29 ΔthyA57 serA endA1 hsdS3

TABLE 1. (Continued)

Strain	Mating Type	Genotype
χ2276	F⁻	dapD22 ΔlacZ39 supE44 Δ47[gal-uvrB] Δ[trpBC] nalA29 ΔthyA57 serA endA1 asd-3 hsdS3
χ2277	F⁻	dapD22 ΔlacZ39 Δ47[gal-uvrB] Δ[trpBC] nalA29 ΔthyA57 serA endA1 asd-3 hsdS3
χ2278	F⁻	dapD22 ΔlacZ39 Δ47[gal-uvrB] Δ[trpBC] nalA29 ΔthyA57 serA endA1 asd-3 hsdS3
χ2279	F⁻	dapD22 ΔlacZ39 Δ47[gal-uvrB] Δ[trpBC] nalA29 ΔthyA57 serA endA1 asd-3 hsdS3
χ2280	F⁻	dapD22 ΔlacZ39 Δ47[gal-uvrB] nalA29 ΔthyA57 serA endA1 asd-3 hsdS3
χ2281	F⁻	dapD22 ΔlacZ39 Δ47[gal-uvrB] nalA29 ΔthyA57 endA1 asd-3 hsdS3
χ2401	pLM2	tonA53 dapD8 minA1 supE42 Δ47[gal-uvrB] minB2 rfb2 nalA25 thyA142 oms-2 metC65 oms-1 Δ29[bioH-asd] cycb2 cycA1 hsdR2
χ2446	F⁻	dapD22 ΔlacZ39 Δ47[gal-uvrB] tyrT58 nalA29 ΔthyA57 serA endA1 asd-3 hsdS3
χ2447	F⁻	dapD22 ΔlacZ39 Δ47[gal-uvrB] tyrT58 nalA29 ΔthyA57 endA1 asd-3 hsdS3

a) The genealogies of the above strains which were constructed in this laboratory are on file with the Coli Genetic Stock Center or can be obtained from R. Curtiss III. None of the strains listed above are lysogenic for λ.

10 g Difco Vitamin Free Casamino Acids to ML or MA (ML+CAA and MA+CAA) contained 0.5% glucose (Glc) and amino acids, purines, pyrimidines and vitamins as required. DL-α,ε-diaminopimelic acid (DAP) was used at 100 μg/ml in all media while generally 20 μg and 40 μg thymidine/ml (Thd) were used in complex and in synthetic media, respectively.

The diluent for bacteria was buffered saline with gelatin (BSG; 9). P1 and λ were diluted in 2.5 mM $CaCl_2$ and 10 mM $MgSO_4$, respectively.

Transduction. P1L4 was propagated on the strains listed in Table 1 by previously described methods (11). Overnight cultures growing in L broth + DAP, Thd containing 2.5 mM $CaCl_2$ were diluted to approximately 1 x 10^7 cells/ml in the same medium and incubated at 37°C with shaking until reaching a density of approximately 2 x 10^8 cells/ml. P1L4 was added at a multiplicity of 1 and allowed to preadsorb at 37°C for 25 min. For experiments in which selection could be provided directly, the preadsorbed mixture was diluted and plated immediately. In cases in which it was necessary to allow for either a segregation or phenotypic lag, the preadsorbtion mixture was diluted one to ten in L broth containing 0.1 M sodium citrate and supplements (minus $CaCl_2$) and the bacteria were allowed to grow to stationary phase before plating.

Direct Selection of Mutants and Mutant Alleles. A spontaneous deletion of the entire gal operon extending through uvrB locus was

isolated by a procedure described by Miller (23) for the isolation of chlorate resistant mutants except that following an initial 24 h of anaerobic growth, the MacConkey-galactose plates were transferred to room temperature and incubated aerobically for an additional 24 h. Isolation with deletions of the gal operon are readily identified by the translucent color and flat spreading morphology of their colonies. Spontaneous nalidixic acid-resistant mutants were isolated by plating concentrated cultures on PA containing 50 μg nalidixic acid/ml.

The ΔthyA57 mutation was introduced into χ1949 by P1L4 transduction and after an appropriate time for segregation lag, the cultures were plated on MA+CAA containing 50 μg thymidine/ml and 10 μg trimethoprim/ml. The plates were incubated at 30°C to eliminate recovery of spontaneous thermosensitive thyA mutants (approximately 30 to 40% of all trimethoprim resistant mutants are thermosensitive) and presumptive ΔthyA57 containing transductants were screened for their non-revertability.

The dapD8 allele was introduced by cotransduction with tonA. After transduction, the bacteria were diluted 10-fold in L broth + DAP containing sodium citrate, grown to stationary phase, plated on L agar + DAP plates prespread with 10^9 T5 bacteriophage/plate and incubated at 37°C.

The supE$^+$ transductants of χ2197 were recovered from a bacterial population which was regrown to stationary phase in L broth + DAP, Thd

containing Na citrate. The stationary phase bacteria were harvested, washed with 10 mM $MgSO_4$, and suspended in an equivalent volume of 10 mM $MgSO_4$. λNNcI857 and λNNcI857·h434 were subsequently added at multiplicities of infection of 5 for a preadsorption period of 25 min at 37°C. The preadsorption mixture was diluted 10-fold in λ broth + DAP, Thd, Mg, shaken continually at 37°C for 12 h and the Mal^+ survivors were tested for their ability to plaque λcI857, λNNcI857 and λNNcI857·h434. These λcI857-sensitive and λNNcI857 and λNNcI857·h434-resistant candidates were mated with χ2401 at a 1 to 1 donor/recipient ratio for one h at 37°C and the Lac^-, kanamycin-resistant, bile salts-resistant transconjugants were tested for their ability to suppress the Ap(am) Tc (am) mutations of pLM2 by plating on PA + DAP containing ampicillin and/or tetracycline at 2.5, 5, 10, 15, 20 and 25 μg/ml. The transconjugants which did not grow at either ampicillin or tetracycline concentrations greater than 5 μg/ml were saved for further transductional investigation. The Su^0 phenotype was assigned to those pLM2 containing candidates which could be simultaneously transduced to both ampicillin and tetracycline resistance by P1L4 grown on χ289 but not by P1L4 grown on χ1918.

The extremely poor growth of χ2277 (Fig. 1) was decreased by transduction of χ2277 by P1L4 (χ1918). This latter transduced population was subjected, as before, to the double λNNcI857-λNNcI857·h434 selection regime, except that the latter cultures were incubated in fully supplemented ML containing 10 mM $MgSO_4$ for 12 h. The largest colonies which grew on fully supplemented minimal media were purified

Figure 1. Genealogy of χ1953, χ2447 and χ2281. The strain numbers in (Table 1) in parenthesis indicate the source of the genes donated. The gene(s) in brackets represent the unselected markers introduced during the procedure. The abbreviation, sel'n. = selection, and amp-cyc refers to ampicillin-cycloserine enrichment (see Materials and Methods). χ2281 was constructed in two steps from χ2279. χ2280 was recovered from the same transduction as χ2446 as a Trp^+ transductant which remained Su^0. χ2280 was transduced to $serA^+$ by P1L4 (χ1753) to yield χ2281.

GENEALOGY

χ1038 (F^-) lacY1 supE44 galK2 galT22 metB1 hsdS3

↓ P1L4 (χ289) with sel'n. for Gal^+

χ1947

↓ Spontaneous with sel'n. for Gal^- Chl^R [attλ-uvrB]

χ1948

↓ Spontaneous with sel'n. for nalidixic acid resistance

χ1949

↓ P1L4 (χ559) with sel'n. for trimethoprim resistance

χ1950

↓ P1L4 (χ289) with sel'n. for Met^+

χ1952

↓ P1L4 (χ1702) with sel'n. for T5 resistance [dapD8]

χ1953

↓ P1L4 (χ573) with Amp.-Cyc. enrichment for Ser^-

χ1954

↓ P1L4 (χ1821) with sel'n. for Ser^+ [endA1]

χ1955

↓ Spontaneous with Amp.-Cyc. enrichment for Ser^-

χ2191

↓ χ341 with sel'n. for Lac^+ [proC $tonA^+$ Dap^+]

χ2192

↓ P1L4 (χ2017) with sel'n. for Pro^+ [ΔlacZ39]

GENEALOGY (continued)

χ2193

↓ P1L4 (χ1633) with Amp.-Cyc. enrichment for Trp⁻

χ2194

↓ P1L4 (χ1702) with sel'n. T5 resistance [dapD8]

χ2195

↓ P1L4 (χ420) with sel'n. for Dap⁺ [pan-tonA]

χ2196

↓ P1L4 (χ2170) with sel'n. for Pan⁺ [dapD22]

χ2197

↓ P1L4 (χ2171) with Amp.-Cyc. enrichment for Thr⁻ met⁻

χ2276

↓ P1L4 (χ1918) with sel'n. for λNNcI857 and λNNcI857·h434 resistance

χ2277

↓ P1L4 (χ1918) with sel'n. for better growth

χ2278

↓ P1L4 (χ1918) with sel'n. for better growth

χ2279

↓ P1L4 (χ2013) with sel'n. for Trp⁺ [tryT58]

χ2446

↓ P1L4 (χ1753) with sel'n. for Ser⁺

χ2447

↓ (F⁻) dapD22 ΔlacZ39 Δ47[gal-uvrB] tyrT58 nalA29 ΔthyA57 endA1 asd-3 hsdS3

and the genotypes of these bacteria were verified. The resulting designate, χ2278, was found to possess a further nutritional requirement which could be satisfied by the water soluble vitamin fraction of an Upjohn multivitamin capsule. Vitamin independent derivatives of χ2278 were selected as papillae on supplemented MA (containing no vitamins) following transduction by P1L4 grown on χ1918.

Ampicillin-Cycloserine Enrichment. The method of Curtiss et al (11) was used to recover mutants or transductants which could not be selected directly. Usually two cycles of enrichment using 100 μg/ml of both ampicillin and cycloserine were sufficient for the recovery of strains with the desired mutations.

Enrichment of dap and asd Mutants. It was necessary to modify the existing enrichment procedures for dap mutants in which protein synthesis was terminated solely by the elimination of DAP (and therefore lysine) from the culture medium (8). The technique devised made use of the observation that the incorporation of DAP into the murein wall is not entirely under stringent control and hence cell wall growth is never completely halted upon the removal of DAP (17). In addition, Dap$^-$ bacteria continue to grow for approximately 0.5 generations on their internal pools of DAP. It therefore was necessary to deplete the bacteria's internal pools of DAP before removing other required amino acids to induce a stringent response. However, a too lengthly depletion of internal DAP pools was detrimental to the recovery of Dap$^-$ mutants since long DAP starvation

in the presence of other amino acids also resulted in loss of Dap$^-$ mutants. Dap$^-$ mutants of χ2087 were recovered by the following method: First, bacteria growing exponentially in fully supplemented ML were deprived of DAP for 2/3 generation to deplete the internal pools of DAP. Second, those amino acids which were present to satisfy the auxotrophy of χ2087 were removed (in this case leucine and arginine) for 40 min. Leucine and arginine were then returned (DAP remained absent) and the cells were incubated in the presence of ampicillin and cycloserine for approximately 3 h. Finally, the antibiotics were removed, the cultures were grown in supplemented minimal, and the entire process was repeated. The Dap$^-$ colonies were scored by replica plating from PA + DAP to PA. Throughout the entire procedure, care was taken to maintain all media at 37^{o}C with filtrations and washes done as rapidly as possible using prewarmed media. In certain instances, homoserine was added and removed concomitantly with DAP to allow the recovery of strains with asd mutations. The recovery of mutants using 2 or 3 cycles of the above regime yielded Dap$^-$ mutants of two basic types. The first and most numerous type of Dap$^-$ mutants was "leaky". The latter Dap$^-$ mutants retained a similar capacity relative to the parent, to synthesize an intact murein and were not investigated further owing to this unacceptable, with respect to safe host construction, phenotype. Ten independent Dap$^-$ mutants which appeared genetically stable were recovered and studied further. The latter mutants unexpectedly fell into two broad groups and the responsible lesions mapped in either the dapD or asd locus.

Propagation of Ch4A. A 0.2 ml sample of a stationary-phase culture of χ1953 or χ2447 previously grown with reciprocal shaking at 37°C in supplemented superbroth was mixed with a total of 1×10^7 pfu of Ch4A which was allowed to preadsorb at 37°C for 20 min. After preadsorption the phage-bacteria mixture was diluted into 15 ml of prewarmed superbroth, a squirt of Dow Antifoam was added and the flask was shaken vigorously at 37°C for 8 h (or until lysis became evident). Chloroform was added and incubation at 37°C was continued for 20-30 min before bacterial debris was removed by centrifugation.

Transfection. The procedures used were kindly made available prior to their publication by G.S. Gill. A stationary-phase culture was used to inoculate 20 ml of L broth to 1×10^7 cells/ml and was shaken at 37°C to a density of 1×10^8 cells/ml. The bacteria were sedimented by centrifugation at 4°C in a Sorval SS-34 rotor at 8000 rpm for 10 min and suspended in 10 ml of cold buffer (50 mM $MnCl_2$, 75 mM $CaCl_2$, 10 mM Na acetate, pH 5.6) for 20 min, concentrated to 0.4 ml and incubated in the same buffer with 100 pg of either Ch4A or λvir DNA for 30 min in the cold. The bacteria-DNA milieu was returned to room temperature for 5 min and immediately plated using λ agar supplemented with DAP, Thd and Mg.

Transduction of Hosts to Lac^+ or Bio^+ by Ch4A. The same conditions outlined above (Propagation of Ch4A) were used except that chloroform was not added at 8 hr. The titers of Ch4A were determined directly on χ1038. The free phages were removed by 3 washings in BSG

before plating for the Lac^+ and Bio^+ transductants on either MA+lactose, Thr, Met, Bio, DAP, Thd or MA+Glc, Thr, Met, DAP, Thd.

DAP-less and Thymineless Death. A stationary-phase bacterial culture growing in L broth + DAP, Thd was used to inoculate 20 ml of L broth + DAP, Thd to $\sim 1 \times 10^7$ cells/ml. The culture was aerated at 37°C and allowed to grow to $2\text{-}4 \times 10^8$ cells/ml at which time the 20 ml were subjected to centrifugation at room temperature in a SS-34 rotor at 11,000 rpm for 2 min, washed with prewarmed BSG (37°C) and the centrifugation repeated. The final pellet was suspended in 2 ml of prewarmed half-strength ML and used to inoculate either prewarmed L broth + Thd, ML + CAA + Bio, DAP or half-strength ML containing 0.1% glucose to the densities shown in the text. Viable counts were determined by diluting in BSG and plating on PA+DAP.

In Vivo Testing in Rats. The procedure of Curtiss et al (13) was followed except that the cultures were grown in L broth + DAP, Thd prior to feeding.

RESULTS

Rationale for the Construction and Selection of χ1038 as the Parental Subline. χ1038 (Table 1), originally isolated by Wood (28), was chosen as the parental strain because it was already in use as a propagating host for some of the proposed EK2 lambda vectors, and was unable to restrict foreign DNA. Mutations contributing to the biological containment of the host were chosen on the basis of their genetic stability and when possible deletions were selected. When deletions were not available, the most stable mutation(s) displaying the desired phenotype were selected. Mutations were considered to be deletions if; i) several closely linked genes simultaneously displayed the mutant phenotype and if their spontaneous reversion frequencies were $\leq 1 \times 10^{-11}$, ii) the mutation did not revert either spontaneously ($\leq 1 \times 10^{-11}$) or after treatment with either chemical mutagens or ultraviolet light exposure. In several cases (*supE*, *nalA* and *hsdS*) the reversion frequency was not calculated because of an inability to score revertants. Data collected during the construction of χ1776 demonstrated that environmental conditions which provided for poor or no growth reduced the ability of χ1776 to undergo DAP-less or thymineless death and thus mutations which contributed auxotrophy were kept to minimum (Curtiss, personal communication).

Construction of χ1953. The derivation of χ1953 is given in Fig. 1. The availability of strong selection techniques permitted the

spontaneous isolation of the Δ47[gal-uvrB] and nalA29 mutations in χ1948 and χ1949, respectively. The ΔthyA57 mutation present in χ1950 provides for thymineless death in environments lacking the pyrimidine and has never been observed to revert (in either the χ1950 or other genetic backgrounds) either spontaneously or after mutagenic treatment (data not shown). The metB1 allele was transduced to wild-type to increase the prototrophic properties of χ1952. The dapD8 mutation was introduced in the final step of the construction of χ1953. At the time of the construction of χ1953, the dapD8 mutation was the most genetically stable dap mutation available.

Construction of χ2447 and χ2281. Incorporated into χ2447 and χ2281 were three mutations which do not directly affect either the safety or utilitarian properties of these strains but which were included in anticipation of modifying χ2279 (see Fig. 1) as a host for plasmid and λ lysogen vectors. The endA mutation introduced into χ1955, see Fig. 1, was observed to increase the transformation efficiency of ColE1-derived plasmids and was therefore introduced at this stage in anticipation of the plasmid hosts. The serA mutation in χ2191 was also introduced to facilitate construction of future hosts with rec and rel mutations. The ΔtrpBC mutation was used to introduce the tyrT58 suppressor into χ2447 and will be used to introduce several markers into future hosts for plasmid and/or λ prophage vectors.

The presence of the lacY1 mutation in hosts for virulent λ vectors was considered disadvantageous for two reasons. First, several λ vectors constructed which contain a functional β-galactosidase (lacZ) gene and thus possessed a significant homology to the lacY$^-$ lacZ$^+$ bacterial chromosome. Second, testing the propensity of a vector to undergo lysogeny and complement the Lac$^-$ defect of bacterial strains possessing the lacY1 mutation could only be undertaken using fermentation media or chromogenic reagents which greatly reduced the sensitivity of the assay.

The introduction of a mutation which deleted the lacZ gene and restored the permease (lacY) activity of the lac operon corrected both of the above problems. The ΔlacZ39 mutation eliminates a portion of the homology that exists between some λ vectors and the E. coli chromosome. The ΔlacZ39 mutation also allowed for the direct quantitation of Lac$^+$ lysogens on minimal lactose medium. The presence of the ΔlacZ39 still allows the detection of inserted DNA fragments in virulent λ possessing lac through the use of techniques outlined by Blattner et al (6).

Numerous attempts were undertaken to transduce the lacY1 mutation directly to lacY$^+$ ΔlacZ39 or lacY$^+$ proC by direct selection on MA + melibiose + supplements at 42^{o}C or on MA + lactose + supplements, respectively. Irrespective of the method or P1 phage employed, no lacY$^+$ transductants were recovered and it became necessary to introduce lacY$^+$ and proC by conjugation into χ2191. The

introduction of the Δ*lacZ39* mutation was carried out by co-transduction with *proC*$^+$.

Much of the concern as to the safety potential of χ1953 rested on its single and revertable *dapD8* mutation. Therefore, much effort was directed towards remedying this defect. The isolation of strains with Dap$^-$ mutations was complicated by the inability to apply conventional enrichment methods for their recovery because of DAP-less death. A successful method was developed (see Materials and Methods) and two of the more stable Dap$^-$ mutations (*dapD22* and *asd-3*) were selected for introduction into the χ1038 subline. The *dapD22* mutation, unlike the *dapD8* allele, does not exhibit a "hazy growth" when plated at high cell concentrations on PA, a phenotype attributed to a limited residual activity of the *dapD* gene product. For the above reasons the *dapD8* mutation was replaced by the newly isolated *dapD22* allele through cotransduction with *pan*$^+$. The TonA$^+$ phenotype was restored to permit infection by the lambdoid bacteriophage, φ80 (or phages possessing its host range), a trait which should facilitate the testing of future vectors. The construction of χ2276 by the inclusion of the *asd-3* mutation effectively eliminates the potential for this host to revert spontaneously to Dap$^+$. The introduction of the *supE*$^+$ allele was accomplished in one step but two further transductions were required to correct the poor growth of χ2277 and χ2278. The cryptic lesion in χ2278 was satisfied by the addition of a water soluble extract (vitamin fraction)

of a commercial multivitamin capsule. The successive transductions of first χ2277 and then χ2278 resulted in a marked increase in generation time and increased the plating efficiency of χ2279 on supplemented MA. χ2447 was constructed in two steps from χ2279. The Ser^- and Trp^- auxotrophy were removed and the tyrT58 suppressor mutation was introduced into χ2447. χ2281 was similarly constructed in two steps from χ2279 except that a Trp^+ transconjugant which remained Su^o (χ2280) was selected for further transduction to Ser^+ to yield χ2281.

Verification of Genotype. The facts that the Dap^- phenotype of χ1953 was introduced simultaneously with tonA during the transduction of χ1952 with P1L4 grown on a tonA53 dapD8 host that the Dap^- mutation was cotransducible with pan and reverted spontaneously at a 5×10^{-7} frequency on PA were taken as proof that the dapD8 allele was present in χ1953. The tonA53 locus was assigned to χ1953 on the basis of its T5 resistant phenotype and because of its simultaneous inheritance with the Dap^- trait in the transduction of χ1952 to χ1953. The supE44 genotype was assigned to χ1953 on the basis of χ2953 susceptibility toward λcI857, λNNcI857, CH3A, CH4A and λgtWES·λB' and its resistance to λcI857 J227 (λcI857 J227 possesses an amber mutation suppressed by tryT but not by the supE cistron). The Δ47[gal-uvrB] genotype was assigned to χ1953 because χ1953 was Gal^- and Bio^- (spontaneous reversion to Gal^+ or Bio^+ was $<1 \times 10^{-11}$) and extremely sensitive toward UV irradiation. Transduction of χ1953 with P1 grown on a host possessing a galK point mutation yielded no Gal^+ transductants and thus indicated that the Gal^- defect extended

into or beyond the galK locus. The nalA29 mutation was assigned by reason of the high (∿75%) efficiency of plating of χ1953 on PA + DAP containing 100 μg nalidixic acid/ml. The ΔthyA57 allele has never been observed to revert and the reversion of Thy⁻ property of χ1953 is quantitatively similar (<1 x 10^{-11}). The ΔthyA57 designation was further strengthened by the inability to recover (<2 x 10^{-10}) Thy⁺ revertants from UV irradiated cultures (sufficient to have caused a 100-fold reduction in viability) of χ1951, a Gal⁺ Bio⁺ UV^R transductant of χ1950. The assessment of the hsdS3 genotype involved two quantitative assays. First, λcI857 grown on modification-proficient (χ289) and modification deficient (χ1038) bacteria plaqued with equal efficiency on χ1953 and second, Ch4A propagated on χ1953 plaqued some 1000-fold less well on χ289 than on χ1038.

The same methods and similar data (with respect to those obtained for χ1953) were recorded for the Δ47[gal-uvrB], nalA29, ΔthyA57 and hsdS3 mutations of χ2447 and χ2281. The Dap⁻ phenotype never reverted to wild-type in either χ2447 or χ2281 (<2 x 10^{-11}). The individual contributions of the asd-3 and dapD22 mutations to the Dap⁻ phenotype were measured separately. The asd-3 allele was tested for its revertability by plating on MA + Glc, Bio, DAP, Thd and reverted at a frequency of 1.8 x 10^{-9}. The dapD22 allele was tested on PA, following transductional removal of the asd-3 mutation by plating cells on PA and reverted at a frequency of 7.7 x 10^{-9}. Interestingly, the dapD22 mutation has been observed to yield "revertants" (10^{-4} - 10^{-5}) if plated on PA supplemented with 0.8% NaCl. The salt-dependent

"revertants", however, could not be purified on DAP-deficient media and therefore remained phenotypically and genotypically Dap$^-$ (Clark-Curtiss, personal communication). No revertants of the Δ*lacZ39* locus were recovered (<1 x 10^{-11}). The Su^0 phenotype was assigned to χ2281 because of its susceptibility to λ*cI857* and its resistance to λ*NNcI857*. The assignment of *tyrT58* to χ2447 was substantiated by the latter's sensitivity toward λ*cI857*, λ*NNcI857* and λ*cI857 J227*. The endonuclease I deficiency (*endA1*) of χ2447 and χ2281 is based on results obtained from the methyl green assay described by Wright (29).

General Properties of χ1953, χ2447 and χ2281. Table 2 compares the generation times of χ1953 and χ2447 under three representative conditions of growth. Paradoxically, χ2447 grew better in less rich media. The small difference in generation time found between χ2447 and χ1953 in ML + CAA medium became exceedingly pronounced in minimal environments.

Although compromised in a variety of ways, χ1953, χ2447 and χ2281 maintained a high susceptibility to λ infection (Table 2) and the plaques of these three strains were slightly smaller in diameter, relative to χ1038, and while clear, showed a slight turbidity at the plaques' extreme peripheral edges. Comparable data for Ch4A are also listed in Table 2 and it was apparent that both χ1953 and χ2447 plaqued Ch4A with equal efficiency. χ2281 by reason of its suppressor-less genotype, was unable to propagate the Ch4A vector. χ2447 pro-

TABLE 2. General properties of hosts

Strain	Generation time (min)[a]			Efficiency of plaquing[b]		Mean yield Ch4A	Transfection
	L Broth	ML+CAA	ML	λvir	Ch4A	(pfu/ml)	frequency[c]
χ1953	53	56	227	0.86	0.88	2.0×10^{10}	1.5×10^{-5}
χ2447	62	51	128	0.90	0.92	2.6×10^{10}	4.0×10^{-5}
χ2281	ND	ND	ND	0.81	$<10^{-8}$	$\leq 10^{1}$	$<10^{-7}$

[a] Generation times were calculated from the increase in absorbancy (A_{620} nm; using a Beckman Spectronic 20) of 10 ml cultures growing at 37°C in either L broth+DAP,Thd, ML+CAA+Bio, DAP, Thd or ML+GLc, Thr, Met, Bio, DAP, Thd in 125 ml side-arm flasks. ND= not determined.

[b] Relative to χ1038

[c] Transfection frequency was calculated as transfectants/Ch4A/genome equivalent. In all cases 1 µg of DNA was taken to equal 2×10^{10} genome equivalents.

duced an average titer of 2.6 x 10^{10} pfu of Ch4A/ml while the same conditions yielded 2 x 10^{10} pfu/ml for the χ1953-Ch4A system, values which are consistent with previous observations (6). It is not necessarily valid to assume that higher titers of Ch4A are not possible for χ1953 since the conditions employed were optimized for χ2447 and could therefore be suboptimal for χ1953.

χ2447 was transfectable at an approximate 3-fold higher frequency than χ1953 using the method of G.S. Gill. Interestingly, more "standard" transfection procedures (5) utilizing magnesium as the principle cation produced similar efficiencies for χ1953 (1.5 x 10^{-5}/ genome equivalent) but showed a reduced efficiency for χ2447 (1 x 10^{-6} /genome equivalent), a phenomenon which emphasizes the need for adjusting transfection conditions to the particular host. Slightly better transfection efficiencies were obtained with λvir DNA (using the manganese method of G.S. Gill) for χ2447 and χ2281 (both ∿ 7 x 10^{-5}/genome equivalent) while χ1953 remained unchanged.

Phenotypic Properties of χ1953, χ2447 and χ2281. The phenotypic properties of χ1953, χ2447 and χ2281 are listed in Table 3. The contributions to the biological safety and utility of hosts possessing the tonA, dapD Δ[gal-uvrB], nalA, thyA and asd mutations have been described previously (13). Both lac mutation present in χ1953 and χ2447 prevent the bacteria from fermenting lactose. The supE44 mutation (SuII) of χ1953 and the tyrT58 mutation (SuIII) of χ2447 allowed for the suppression of genes containing amber mutations. Three enzymes active on DNA are eliminated or inactive because of the

endA1 mutation of χ2447 and χ2281 and/or the hsdS3 mutation found in all three strains. The endonuclease I (endA) activity associated with the periplasmic space of E. coli was not present in χ2447 and χ2281. The hsdS3 mutation removed the substrate specificity of the K-12 restriction-modification multimeric complex producing the $r^- m^-$ phenotype found in all three strains.

There are several phenotypes for which the mutational lesion(s) was (were) not identified. Chronologically the first to appear was the P1 resistance and bile salts sensitivity of the host. The combination of phage resistance and detergent sensitivity has been reported previously (20) and the causative defect(s) was (were) nearly always shown to be the consequence of an altered cell wall in which phage resistance resulted from an inability of the phage to adsorb and/or inject its genome. A similar explanation was tested for χ1947 but it was evident that a block at the level of adsorption or penetration could not explain the simultaneous appearance of phage resistance and bile salts sensitivity. The efficient recovery ($\leq 1 \times 10^{-5}$) of transductants from χ1947 clearly indicated that deficiency in production of P1 progeny was best interpreted as a block in some later infection event, i.e. replication or phage packaging. The presence of a prophage possessing a P1-like immunity system was compatible with the high frequency of transduction of χ1947 and would have explained the lack of P1 progeny following infection. Neither temperate, P1kc, nor the independently derived virulent mutants P1L4 and P1vir, plaqued on χ1947 or its progeny, thus a

TABLE 3. Phenotypic properties of χ1953, χ2447 and χ2281

Properties	Responsible Mutation	
	χ1953	χ2447[a]
Resistant to T1, T5, ϕ80	tonA53	-
Requirement for DAP	dapD8	dapD22 asd-3
Unable to ferment lactose	lacY1	ΔlacZ39
Suppress amber mutations	supE44	tyrT58
Unable to ferment galactose	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Requirement for biotin	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Extreme UV sensitivity	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Prevents exopolysaccharide synthesis	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Resistant to nalidixic acid	nalA29	nalA29
Resistant to trimethoprim	ΔthyA57	ΔthyA57
Requirement for thymine or thymidine	ΔthyA57	ΔthyA57
Requirement for threonine and methionine	-	asd-3
Deficient in endonuclease I	-	endA1

TABLE 3 (continued)

Properties	Responsible Mutation	
	χ1953	χ2447[a]
Deficient in restriction and modification	hsdS3	hsdS3
Resistant to P1	?[b]	?[b]
Sensitive to bile salts	?[b]	?[b]
Requirement for higher than normal levels of thymine or thymidine	-	?[b]

[a] χ2281 possesses all the mutations and phenotypes found in χ2447 except that it (χ2281) is tyrT$^+$ (Su^0)

[b] The genetic lesion responsible for the phenotype is not known.

prophage, if present, would be expected to be deleted for those regions affecting immunity. Similarly, χ1947 plaqued λ with a ≥ 90% efficiency which provided evidence that χ1947 did not harbor a prophage possessing a P1-like restriction-modification system (2,7). Finally, plasmids were not detected in χ1947 by using either ethidium bromide-CsCl or alkaline sucrose gradients (data not shown). Several spontaneous bile salts-resistant mutants of χ1953 and χ2281 were tested and found to retain their refractiveness to P1 infection. It was not established that these mutants represent revertants of the original lesion, for various morphological classes of resistant clones were recovered and a further testing of these classes was not attempted.

It was possible that the P1 resistance and/or bile salts sensitivity present in χ1947 was introduced during the transduction of χ1038 to Gal^+ by P1 grown on χ289 (neither χ1038 nor χ289 express P1 resistance or bile salts sensitivity). χ1038 was again transduced to Gal^+ by P1 grown on χ289 and the Gal^+ transductants were scored for their ability to plaque P1 and their sensitivity toward 0.15% bile salts. None of the 109 Gal^+ transductants tested were $P1^R$ and/or bile salts sensitive.

Both χ2447 and χ2281 plated with an approximately ten-fold lower efficiency on PA + DAP + bile salts (0.15%) than the already decreased χ1953 (efficiency of plating relative to χ1038 of 0.004). The defect responsible for the increased bile salts sensitivity of

χ2447 compared to χ1953 was traced to a predecessor of χ2276 (Fig. 1) but the exact step was not ascertained. Visible clumping was observed for bacteria grown in L broth + DAP, Thd commencing with the construction of χ2194 (Fig. 1), as coincidentally with the increased requirement for thymidine. The requirement of χ1953 for thymidine or thymine was satisfied by L broth + DAP as it is for most strains possessing the ΔthyA57 mutation. χ2447 and χ2281 possessed an additional defect that caused these bacteria to require that L broth + DAP be additionally supplemented with 10 μg Thd/ml. In the absence of such supplementation the titers of cultures seldom reached $> 5 \times 10^7$ cells/ml and microscopically the bacteria appeared as long filaments. Both lower titers and filamentous growth are characteristics of thyA mutants grown in suboptimal concentrations of thymine (4,22).

Assessment of the Probability for the Stable Inheritance (Transduction) of Genetic Information from Ch4A. Ch4A has inserted into its genome two convenient markers. The lac5 insertion includes a functional lac promoter and lacZ genes and allows for direct complementation of the ΔlacZ39 deletion in χ2447-χ2281 and for an indirect assay in χ1953 (6). Ch4A also possesses a bio insertion which complements the Bio^- defect of χ1953, χ2447 and χ2281. Table 4 summarizes the results of experiments performed to assess the potential for transduction in our hosts. The absence of suppresssor mutations in χ2281 did not favor either Lac^+ or Bio^+ transduction (2.1×10^{-6} and 2.8×10^{-5}/surviving bacteria, respectively) and a

TABLE 4. Assessment of the probability of Lac^+ and Bio^+ transduction by Ch4A[a]

Strain	Transductants/surviving bacterium		Transductants/output Ch4A[b]	
	Lac^+	Bio^+	Lac^+	Bio^+
χ1953	1.7×10^{-3}	3.1×10^{-3}	5.9×10^{-7}	1.1×10^{-6}
χ2447	2.0×10^{-3}	1.1×10^{-4}	1.3×10^{-6}	9.5×10^{-8}
χ2281	2.1×10^{-6}	2.8×10^{-5}	2.4×10^{-3}	3.2×10^{-2}

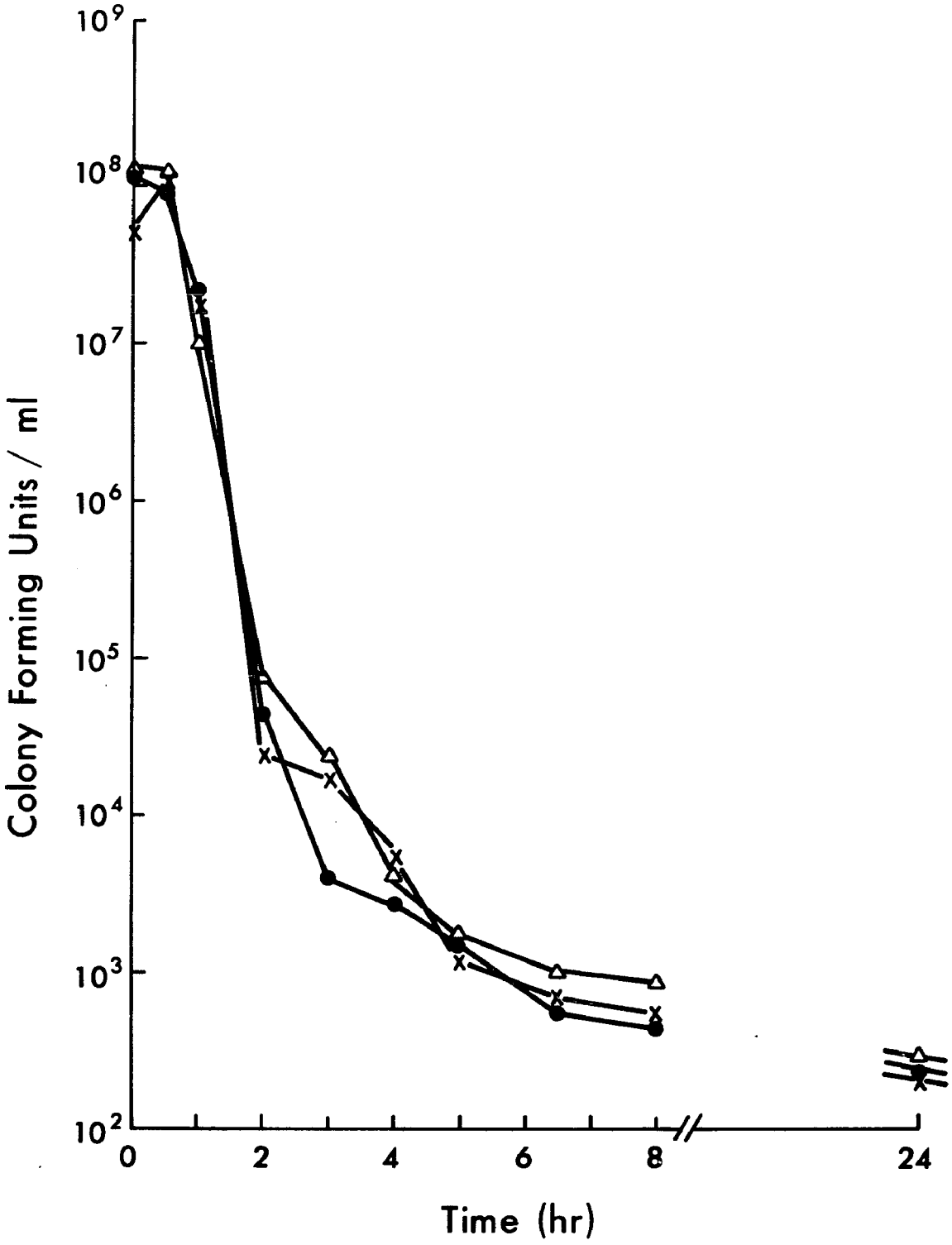
a

The transductants were collected after 8 h of incubation in the presence of Ch4A and were sedimented by centrifugation and washed repeatedly with BSG. The successive washes were sufficient to have reduced the unbound Ch4A by 10^6-fold. The final bacterial suspension was concentrated 5-fold before plating on MA + Lac, Thr, Met, Bio, DAP, Thd or MA + Glc, Thr, Met, Dap, Thd. The titers of the surviving bacteria (concentrated) were 7.8×10^7, 1.3×10^8 and 3.2×10^9 for χ1953, χ2447 and χ2281, respectively.

b

The input of Ch4A was 6.8×10^5 pfu/ml and the output of Ch4A after 8 h for χ1953, χ2447 and χ2281 was 4.4×10^{10}, 4.0×10^{10} and 5.6×10^5 pfu/ml, respectively.

Figure 2. Kinetics of DAP-less death in L broth. A stationary culture of each strain was used as the inoculum of L broth+DAP+Thd and each of these cultures were grown with shaking at 37°C until reaching a density of approximately $2x10^8$ cells/ml. The bacteria were then sedimented by a brief (2 min) centrifugation in a Sorval SS-34 rotor at room temperature and washed in prewarmed BSG (37°C) before resedimenting as before. The resulting bacterial pellet was suspended in 0.1 volume (relative to initial volume of bacterial culture) of prewarmed BSG and used to inoculate fresh prewarmed (37°C) L broth + Thd. The DAP-free cultures of χ1953 (●) χ2447 (Δ) and χ2281 (x) were titerd on PA + DAP, Thd at the times depicted in the figure.

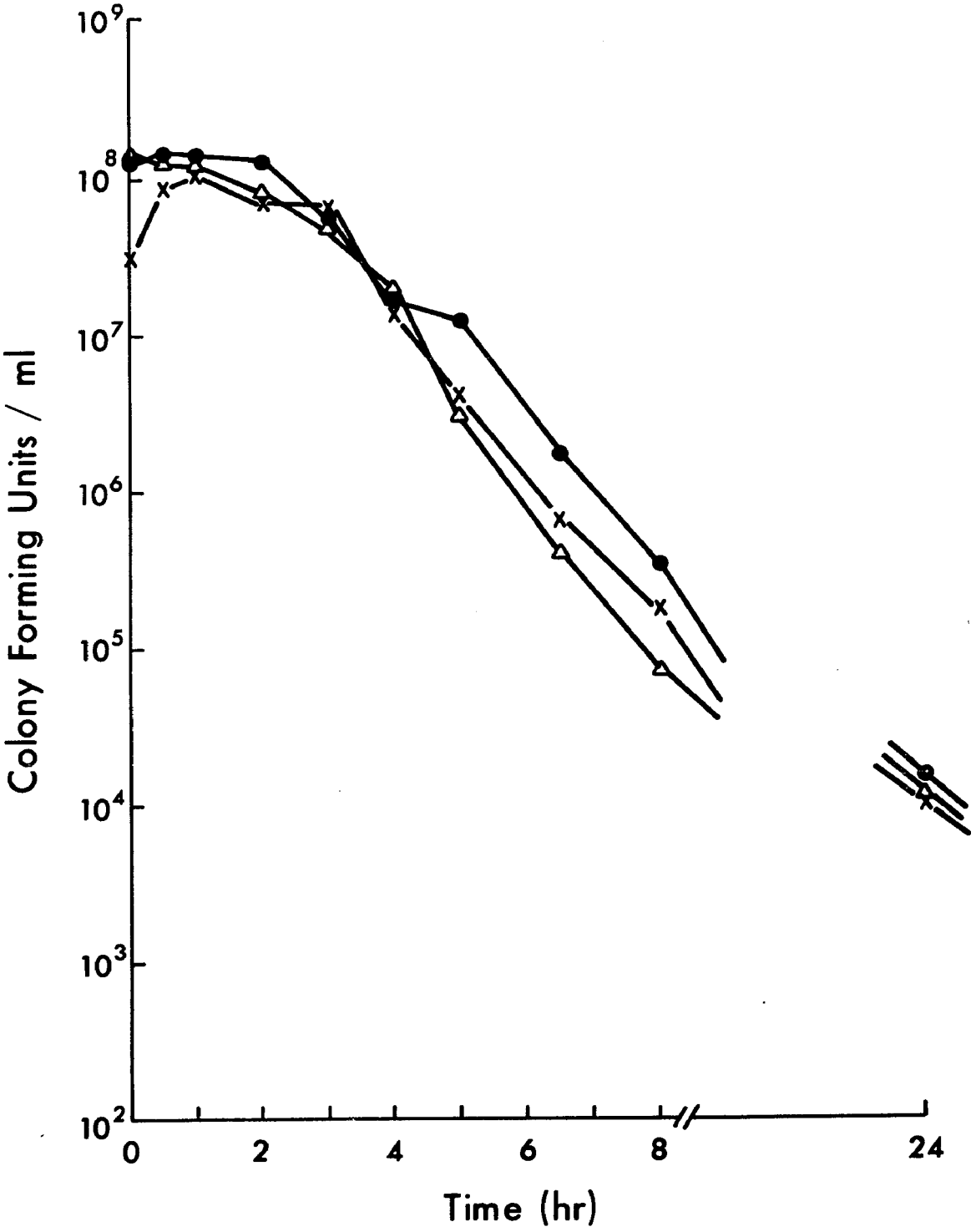

Colony Forming Units / ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
24
Time (hr)

significant decrease in infective centers was seen by 8 h. The Lac^+ and Bio^+ transductants were purifiable on lactose minimal and minimal medium lacking biotin, respectively.

Survival in the Absence of Diaminopimelic Acid. Fig. 2 records the rates and extents at which χ1953, χ2447 and χ2281 underwent DAP-less death in L broth + Thd. The bacteria shortly following the initial stasis (∿ 30 min) entered a period of rapid and exponential suicide with survival decreasing at an apparent half life ($t_{½}$) of 6.7, 8.5 and 6.3 min for χ1953, χ2447 and χ2281, respectively, Macroscopically this phase of DAP-less death was accompanied by a rapid decrease in the absorbance of the culture and an increase in viscosity of the medium due primarily to the release of DNA into the culture medium. Continued incubation led to further cell death, albeit, at a reduced rate, achieving an approximately 5×10^5-fold reduction in viable count by 8 h for each host.

Survival in the Absence of Thymine. χ1953, χ2447 and χ2281 (Fig. 3) were assessed for their ability to survive in growth media lacking thymine or thymidine. After a short lag of ∿ 1 h, a delay at least partially explainable as that time necessary for the bacteria to deplete their internal pools of the essential nucleotide, the bacteria commenced thymineless death. Once initiated, thymineless death in these hosts proceeded rapidly. There was little significant difference in the rate and/or extent at which χ1953, χ2447 and χ2281 survived when deprived of thymidine, as 8 h under these conditions

Figure 3. Kinetics of thymineless death. The same washed suspensions of χ1953 (●), χ2447 (Δ) and χ2281 (x) used to inoculate the DAP-deficient L broth described in Fig. 2 were used to inoculate ML + CAA, Bio, DAP. Titers were determined as described in the legend to Fig. 2.

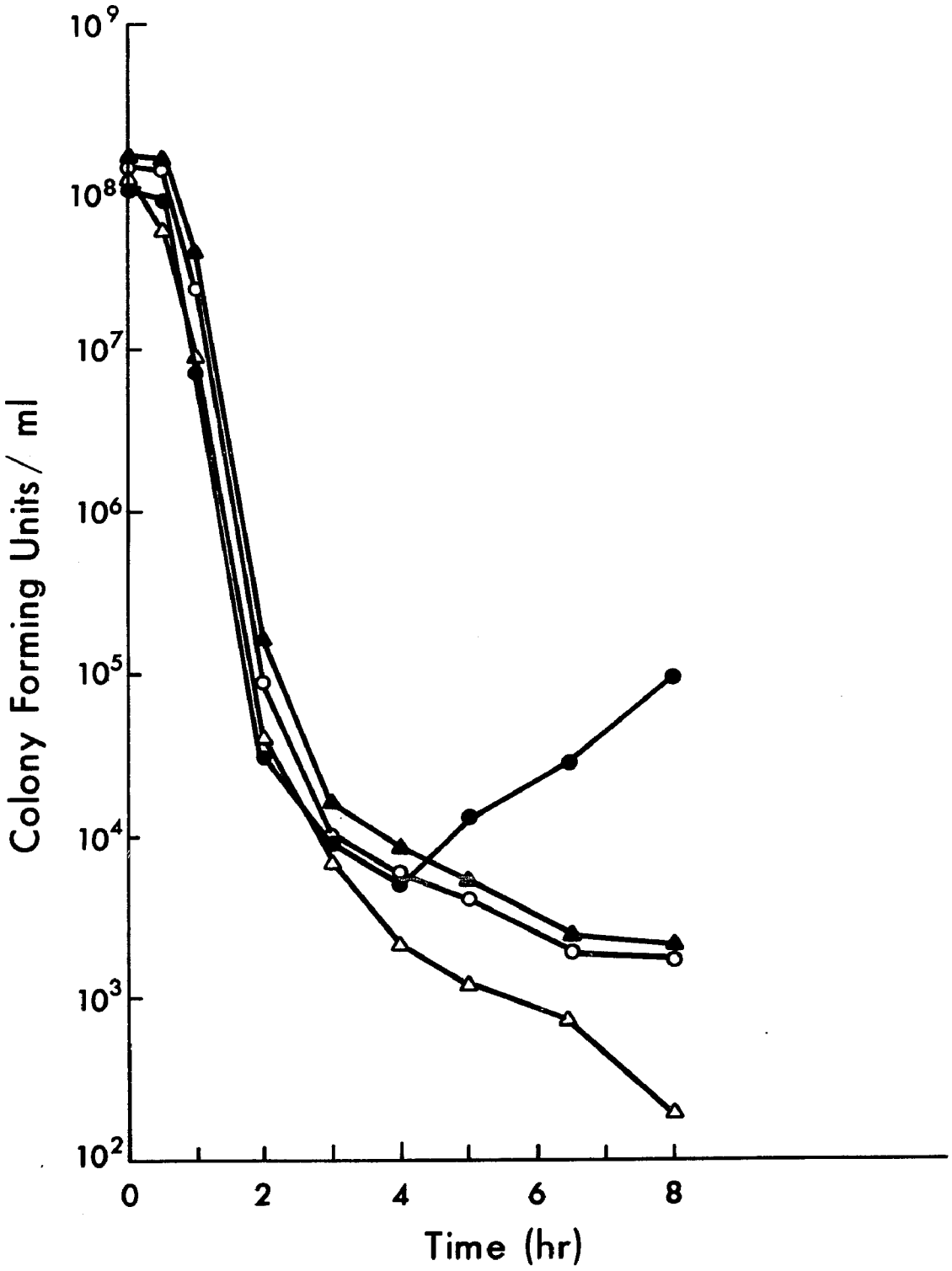

Colony Forming Units / ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
24
Time (hr)

was sufficient to have caused a 3 x 10^2-, 1 x 10^3-, and 5 x 10^2-fold reduction in titers for χ1953, χ2447 and χ2281, respectively. Though the rates for thymineless death decreased after 8 h, it was evident that death continued through at least 24 h of starvation.

DAP-less Death of χ1953 and χ2447 from Overgrown Ch4A Lysates. In Fig. 4, two distinct survivors (distinguished from each other by their colony morphology on PA + DAP) of χ1953 and χ2447, recovered from overgrown 24 h lysates of Ch4A, were tested for their ability to undergo DAP-less death. The kinetics of death were virtually identical to those obtained for the DAP-less death of χ1953 and χ2447 reported in Fig. 2. Both χ1953 survivors underwent DAP-less death at $t_{½}$ of 7.4 min while the χ2447 survivors had $t_{½}$ of 7.4 and 7.6 min. The data in Fig. 2 also illustrates that the single Dap^- mutation in χ1953 was sometimes observed to lead to the predomination of Dap^+ revertants in cultures which had been starved for DAP for as little as 4 h.

Survival in Conditions Simulating Those Possible in Nature. In testing the survival properties of a host, one is presented with the task of envisioning various potential environmental conditions that the host and its vector might encounter should they escape the confines of the laboratory. Taken literally one would feel obliged to test virtually thousands of conditions for the survival potential of each host. Conditions were chosen which simulate probable routes of escape, i.e. oral ingestion and release into the waterways.

Figure 4. Kinetics of DAP-less death in L broth for survivors of Ch4A infection. The morphologically distinct survivors (surviving Ch4A infection) of χ1953 (o,●) and χ2447 (Δ,▲) were assayed for their ability to undergo DAP-less death in L broth + Thd as described in Fig. 2.


10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
Colony Forming Units / ml
0
2
4
6
8
Time (hr)

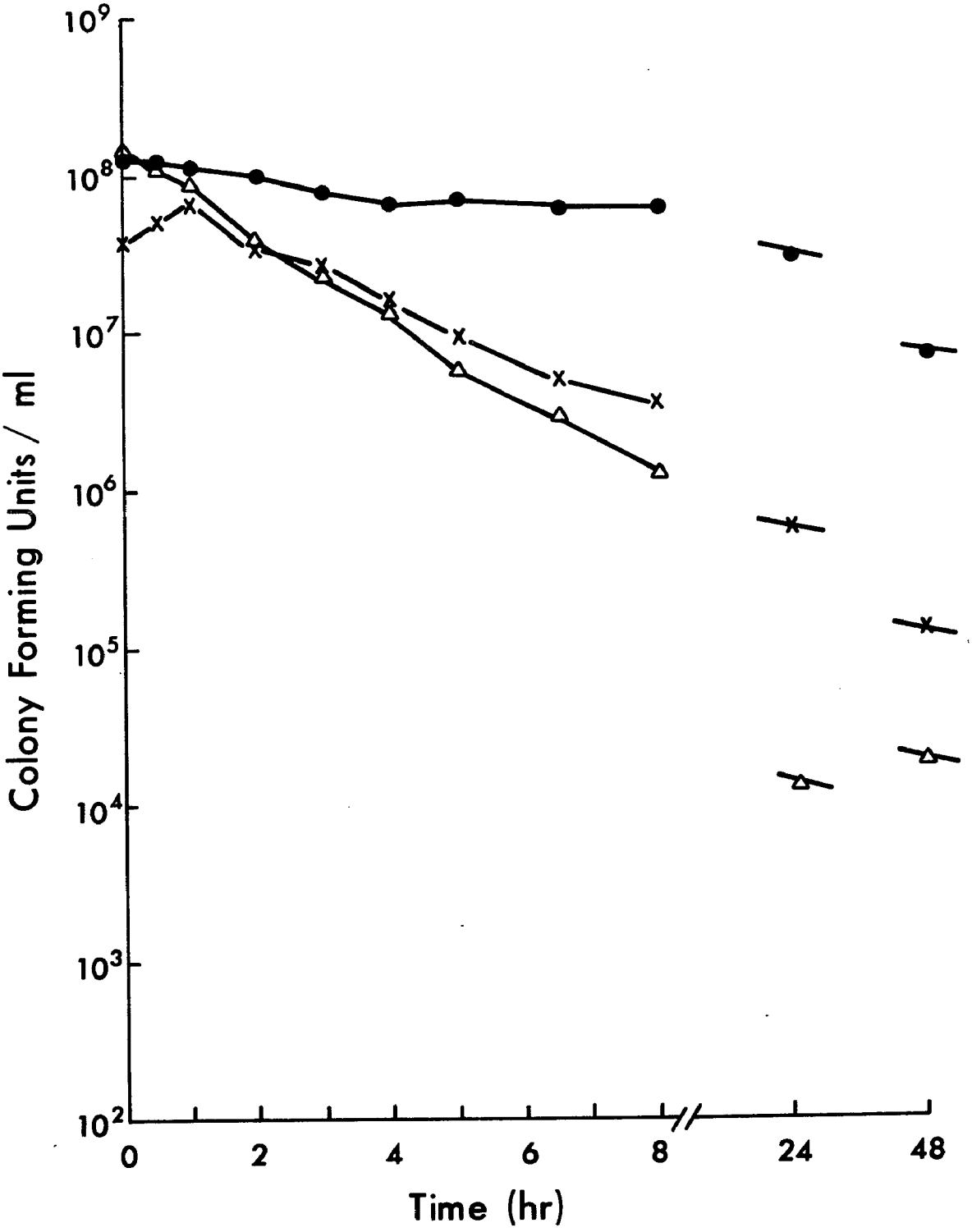
Survival following oral ingestion was studied in rats as described in Materials and Methods. χ2100 (χ1038 Nal^r) and χ2447 were recovered from the feces of the animals for 72 h after feeding, while χ1953 was detected only for 48 h (Table 5). Suprisingly, χ2281 survived less well than its isogenic sibling, χ2447, being eliminated by 18 h from the alimentary canal of the rodents. The quantity of bacteria surviving passage for χ2281 was also markedly lower than any of the other strains tested. Experiments with gnotobiotic mice confirmed these data for χ1953 with the interesting discovery that χ1953 is also unable to colonize the same gnotobiotic animals (R. Freter, personal communication).

The in vitro aquatic system employed contains a modest complement of inorganic salts together with a limited carbon supply (lacks DAP and Thd). Fig. 5 illustrates the considerable difference that was found in the behavior of χ1953 vs. that of χ2447 and χ2281 when incubated in this aquatic system. The contrast was striking with approximate $t_{½}$ values for χ1953, χ2447 and χ2281 of 480 min, 71 min and 98 min for the initial 8 h of incubation. Interestingly, further exposure to this environment provided approximately equal rates of cell death for all three strains.

TABLE 5. Survival in rats following oral administration

Strain	No. Rats Tested	Total Bacteria Fed	Mean surviving bacteria/ml/0.1g feces at designated hour after feeding							
			0	6	12	18	24	48	72	96
χ2100	6	1.5×10^{11}	≤4	3×10^{6}	4×10^{6}	2×10^{6}	9×10^{5}	2×10^{3}	2×10^{2}	≤4
χ1953	3	6.1×10^{10}	≤4	1×10^{5}	2×10^{5}	4×10^{3}	4×10^{3}	1×10^{1}	≤4	≤4
χ2447	6	4.8×10^{10}	≤4	2×10^{4}	7×10^{6}	3×10^{6}	9×10^{4}	1×10^{2}	2×10^{2}	≤4
χ2281	3	1.7×10^{10}	≤4	1×10^{5}	7×10^{3}	4×10^{1}	≤4	≤4	≤4	≤4

Figure 5. Survival of χ1953, χ2447 and χ2281 in a simulated aquatic environment. The same washed suspensions of χ1953 (•), χ2447 (Δ) and χ2281 (x) described in Fig. 2 were used to inoculate half strength ML containing 0.1% glucose. The cultures were titered on PA + DAP, Thd.


Colony Forming Units / ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
24
48
Time (hr)

DISCUSSION

The ideal biologically compromised host would grow with a generation time equal to that of wild-type bacteria, transfect or transform at higher efficiencies than wild-type hosts, be compatible with the current and future methodologies practiced in recombinant DNA research, and yet be sufficiently enfeebled to guarantee the timely and rapid demise of the host and its vector under all conceivable conditions that might be encountered in the biosphere. Unfortunately, very few mutational lesions can be introduced into any bacterium without the loss of some of the cell's original vitality. Often mutations which only minimally affect the robustness of a bacterium, will, when in combination with other similarly "benign" mutations, be synergistic to reduce vitality. Thus in practice, the construction of biologically contained systems focuses on the balancing of the bacterium's utility and safety properties.

This report details the construction of a host, χ1953, certified as EK2 when used in conjunction with certain λ vectors and further reports the development of a host-lambda system which appears to surpass the currently certified, χ1953, in both safety and utility. Three genetic techniques, transduction, spontaneous mutant selection, and conjugation were used in the construction of χ1953, χ2447 and χ2281. Most of the mutations contained in these hosts were introduced through P1 transduction and because of the inability to select directly for certain of the introduced lesions,

other mutations not present in the final derivatives were introduced solely for the purpose of strain construction. The direct selection of spontaneous lesions was the method of choice for those mutations which were amenable to this technique and in one instance markers were introduced via conjugation. In no instance were mutagenic agents, chemical or radiomimetic, directed toward any of the intermediates of these hosts and similarly temperate bacteriophages and conjugative plasmids were also shunned in the constructions. Thus, the inadvertent lysogeny or introduction of cryptic plasmids and/or portions of the latter replicons into these hosts has been eliminated.

The safety and utility properties of χ1953, χ2447 and χ2281 (Table 6 and Table 7) can be categorized into: (i) those that effect the bacterium's cell wall (due to the tonA, dapD, and Δ[gal-uvrB] mutations; χ2447 and χ2281, in addition possess the asd mutation), (ii) those which allow for testing (the Δ[gal-uvrB] mutation; in addition, χ2447 and χ2281 possess the ΔlacZ mutation and χ2281 has a Su^0 phenotype) and monitoring due to the nalA and ΔthyA mutations), (iii) those related to the DNA synthesizing machinery of these cells (due to the ΔthyA57 and hsdS mutations), and (iv) those miscellaneous mutations affecting ultraviolet light sensitivity (Δ[gal-uvrB]), P1 resistance, bile salts sensitivity, and a high thymidine requirement in which the specific mutational defect(s) was(were) not always known.

It was surprising that the introduction of the gal^+ operon

TABLE 6. Utility properties of χ1953, χ2447 and χ2281

Property	Responsible mutations in	
	χ1953	χ2447[a]
Propagation of vectors with SuII suppressible mutations	supE44	tyrT58
Propagation of vectors with SuIII suppressible mutations	-	tyrT58
Highly transfectable	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Restrictionless	hsdS3	hsdS3
Monitor vectors	lacY1	ΔlacZ39
	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Improved growth in minimal nutrient conditions	-	?[b]
Efficient labeling of DNA	ΔthyA57	ΔthyA57
Efficient labeling of proteins	Δ47[gal-uvrB]	asd-3
		Δ47[gal-uvrB]

TABLE 6 (continued)

[a] χ2281 possesses all the attributes of χ2447 except by reason of its Su^0 phenotype is unable to propagate vectors possessing amber mutations. The Su^0 phenotype permits χ2281 to be used as a highly contained testing strain for the verification of the amber mutations contained on the vectors.

[b] The genetic modification responsible for the increased growth in minimal media is not known but is lacking in χ1953.

TABLE 7. Safety properties of λ hosts

Properties	Responsible mutation	
	χ1953	χ2447[a]
DAP-less death	dapD8	dapD22 asd-3
Unable to synthesize an exopolysaccharide	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Increased sensitivity to UV	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Deletes attλ to reduce lysogeny by λ	Δ47[gal-uvrB]	Δ47[gal-uvrB]
Thymineless death	ΔthyA57	ΔthyA57
Decreases the probability of inadvertant contamination[b]	nalA29 ΔthyA57	nalA29 ΔthyA57
Resistant to P1	?	?
Sensitive to bile salts	?	?[c]
Increased requirement for thymidine	-	?[d]

[a] The same properties apply to χ2281

TABLE 7 (Continued)

[b] The likelihood of bacterial contamination is reduced if the culture media is supplemented with nalidixic acid and/or trimethoprim at 25 μg and 10 μg/ml, respectively. The *nalA29* and Δ*thyA57* mutations provide a high level of resistance to the above antibiotics.

[c] χ2447 and χ2281 have an additional sensitivity to bile salts over that found in χ1953, however, χ1953 is likewise more sensitive than its predecessor, χ1038.

[d] The nature of the genetic lesion has not been fully characterized but the phenotype is missing in χ1953.

caused χ1947 to express P1 resistance and an increased bile salts sensitivity (Fig. 1). Three general models might account for the simultaneous appearance of P1 resistance and bile salts sensitivity during the introduction of the wild-type gal genes into χ1038. First, the phenotypes of P1 resistance and bile salts sensitivity were not related, and the P1 resistance exhibited by χ1947 was the consequence of lysogeny. Second, a cryptic lesion contained in χ289 and cotransducible with the gal locus became expressed upon its introduction into χ1038. Third, it is possible that χ1038 accumulated secondary suppressor mutations which were incompatible with the wild-type gal alleles.

The genes comprising the immunity system of P1 are distributed in two physically separated regions of the genome (30). The genes which encode for the restriction-modification enzymes of the phage are also distinct (30). Neither of the latter P1 functions were detected in χ1947 or its progeny and a prophage, if present, would by necessity be severely deleted so as to possess neither property. Further no extrachromosomal DNA was detected in χ1947. Finally, the probability of recovering a Gal^+ transductant which had simulutaneously inherited a defective prophage is remote and the existing evidence does not substantiate model one.

Model two appears unlikely as no $P1^R$ and/or bile salts sensitive transductants were recovered from the Gal^+ transductants of χ1038 using P1 grown on χ289. It remains possible that such a cryptic lesion as

model two presupposes is present in χ289 and the inability to detect its cotransduction with the introduction of Gal^+ was the consequence of the number of Gal^+ transductants tested (109 Gal^+ transductants).

It is not possible at this time to eliminate model three, however, it is interesting to speculate that a single lesion is responsible for both phenotypes, possibly interacting with the cytoplasmic membrane (but not necessarily restricted solely to the inner membrane) in such a manner that late events in phage infection, i.e. phage replication or packaging are prevented and that the bile salts sensitivity exhibited by these strains, results through the indirect interaction of the defective cytoplasmic membrane with the other cell layers.

Another unexplained phenotoype was the increased requirement for externally added thymine and/or thymidine which appeared in the construction of χ2194 (Fig. 1) and which is, therefore, absent in χ1953. A thymine permease activity is unknown in E. coli but a nucleoside transport system has been reported, and thus the increased need for thymine and/or thymidine was most probably the result of a decrease in permeability toward the former pyrimidines (24). There are several reports in which the entrance of thymidine into the cell was shown to be a consequence of outer membrane perturbations (14), indeed cultures of χ2194 and its progeny, including χ2447 and χ2281 exhibited an increased tendency toward aggregation and clumping, a phenotype suggestive of surface changes.

Possibly related to the preceding phenomenon is the paradoxic growth of χ2447 in L broth + DAP, Thd vs. ML+CAA, Bio, DAP, Thd (Table 2). χ2247 grew with a generation time of 62 min in L broth plus supplements but surprisingly showed a decrease in generation time to 51 min in ML+CAA, Bio, DAP, Thd. χ1953 on the other hand exhibited a progressively decreased generation time as the "richness" of the medium was decreased. The mechanism or cause(s) of the latter phenomenum is not understood but it is likely to be related to the same changes in the genetic make-up of the bacteria which cause the increased requirement for thymidine.

The χ2447-χ2281 system possesses several properties which are superior to the χ1953-host system and is compatible with all currently certified λ vectors (Table 6). χ2447 not only produces high yields of Ch4A but is also highly transfectable. The testing of future vectors was also simplified by the inclusion of mutations which allowed for the direct assessment of phage encoded complementation. It is possible to efficiently label the DNA of lambda-infected hosts, χ1953, χ2447 and χ2281 by the addition of radiolabeled thymine or thymidine since all three strains are lacking a thymidylate synthetase activity. The χ2447-χ2281 host system offers the unique advantage of possessing an auxotrophic requirement for threonine and methionine and thus provides the investigator with an additional ability to label vector-encoded proteins. In this regard, χ2281 is particularly useful for its Su^0 phenotype prohibits transcription and subsequent translation of those phage proteins distal to the nonsense codons

contained in the A and B cistrons and therefore provides for an enrichment of the cloned products. Two other procedures can provide still further recoveries of the proteins encoded by the cloned fragments. The first makes use of the ability to insert the foreign DNA segment into the lac5 insertion of Ch4A, while maintaining the capability of initiating transcription from the lac promoter (for further discussion see reference 6). The second property is provided by the host itself. The presence of the Δ47[gal-uvrB] mutation endowed each host with an extreme sensitivity to ultraviolet light and the judicious use of this bactericidal property can minimize the synthesis of proteins of bacterial origin (16).

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CHAPTER II

INTRODUCTION

The murein layer of E. coli is loosely attached to the outer membrane through its covalent linkage with the lipoprotein first described by Braun, and the hydrophilic interaction of the latter lipoprotein with the lipid bilayer of the outer membrane and the electrostatic and hydrophobic interactions of the murein associated matrix proteins (4,7,8,20). The murein, however, exerts its primary influence by functioning as a molecular sieve and provides the necessary rigid netting around the osmotically fragile cytoplasmic membrane. The murein layer of E. coli has been extensively studied and is characterized by: (i) a repeating disaccharide polymer backbone of N-acetyl glucosamine and N-acetyl muramic acid linked β1-4, (ii) a tetrapeptide composed of L-alanine D-glutamicacid, diaminopimelic acid (DAP) and D-alanine extending from the sugar backbone, and (iii) a peptide bond linking two different tetrapeptides (4,8,20).

E. coli produces a more distal (relative to the outer membrane) layer composed of colanic acid under certain conditions of stress (nutritional starvation, or phage and antibiotic exposure) or through mutations in the capR, capS, or capT loci (1,2,9,16,17,26). The secreted exopolysaccharide is unique among the greater than 100 K(capsular) antigens described for other E. coli strains primarily through its lack of an apparent linkage with the outer membrane (13). Operationally, colanic acid can be isolated directly from the super-

natant liquid of cultures secreting the exopolysaccharide. Capsules require only a mild phenol pretreatment in order to accomplish these same sorts of recovery and thus colanic acid is most often described as a slime or M-antigen to emphasize this distinction (14). Another characteristic of colanic acid that sets it apart from K antigens is the ubiquity of the same exopolysaccharide in various species of Enteriobacteriacae (2,11,12). Chemically, colanic acid is a repeating hexasaccharide composed of galactose (Gal), glucose (Glc), glucuronic acid (GlcA) and fucose (Fuc) in ratios of 2:1:1:2 which may be acetylated at the primary fucose and/or possess a variety of ketal substituents at the terminal galactose moiety (10,11,22,23).

Cell-free synthesis of colanic acid in capR mutants of E. coli revealed that the nucleotide monosaccharides are apparently added sequentially to the C-55-isoprenoid lipid carrier (IPP) in the formation of colanic acid (15). The isolation of an (Fuc_3, Glc_2)-Glc-IPP intermediate in these extracts has led to the postulation that as in O-antigen synthesis, the backbone oligosaccharide →Fuc-Fuc-Glc→ may be joined separately to the branching Gal-GlcA-Gal trisaccharide via a separate IPP-trimer to yield the final hexameric form, rather than through an entirely sequential synthesis as was demonstrated for exopolysaccharide production in Klebsiella (24). Various mutants defective in murein synthesis or septation were shown to produce colanic acid (21,25) and it has been suggested that the IPP intermediate coordinates the synthesis of murein, LPS and colanic acid (23).

The production of a capsule endows the bacterial cell with several properties including; (i) phage resistance owing to the inability of certain phage to reach their respective receptors in the outer membrane, (ii) antibody and complement resistance, (iii) increased invasiveness, and (iv) a postulated but unproven resistance to desiccation through the storage of water in the highly hydrated slime (approximately 99% hydrated) (9). A fifth property of colanic acid is described in this report.

It was expected that the elimination of a bacterium's ability to synthesize diaminopimelic acid (DAP) by growing the bacterium in DAP-deficient media would result in the ultimate death of every member of Dap^- population. However, E. coli was found to possess the ability to circumvent mutational blocks in its DAP biosynthesis by secreting an exopolysaccharide and not only survive but increase in titer, provided the correct ionic environment was maintained.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this report are listed in Table 8. The gene symbols are those suggested by Bachmann et al. (3) and allele numbers were assigned by the Coli Genetic Stock Center.

Media and Chemicals. All media were as described in Chapter 1 except D broth (10 g Difco Tryptone/l, 5 g Difco Yeast Extract/l) and D agar (D broth with 15 g Difco agar/l). Materials for chromotography were the kind gift of J. Schutzbach.

DAP-less Death. L broth + Thd, DAP was inoculated from stationary-phase cultures grown in the same medium to approximately 1×10^7 cells/ml, and these cultures were shaken at 37°C until reaching a density of approximately $1\text{-}2 \times 10^8$ cells/ml. The exponentially growing bacteria were sedimented by centrifugation at 10,000 rpm in a Sorval SS-34 rotor at room temperature and washed with prewarmed (37°C) BSG, the centrifugation was repeated and the final pellet suspended in BSG. The BSG suspension was immediately inoculated into prewarmed L broth + Thd, D broth + Thd (adjusted to the salt concentration described in the figures) or ML+CAA, Bio, Thd to the density shown in the figures, incubated at 37°C with shaking, and sampled for viability by plating on PA + DAP.

TABLE 8. Bacterial strains

Strain number	Mating Type	Genotype
χ1846	F⁻	tonA53 dapD8 minA1 purE41 supE42 minB2 his-53 nalA25 metC65 Δ29[bioH-asd] ilv-277 cycB2 cycA1 hsdR2
χ1849	F⁻	tonA53 dapD8 minA1 purE41 supE42 Δ40[gal-uvrB] minB2 his-53 nalA25 metC65 Δ29[bioH-asd] ilv-277 cycB2 oms-1 cycA1 hsdR2
χ1776	F⁻	tonA53 dapD8 minA1 supE42 Δ40[gal-uvrB] minB2 rfb-2 nalA25 thyA oms-2 metC65 oms-1 Δ29[bioH-asd] cycB2 cycA1 hsdR2
χ2447	F⁻	dapD22 ΔlacZ39 Δ47[gal-uvrB] tyrT58 nalA29 ΔthyA57 endA1 asd-3 hsdS3

Chemical Analysis of Exopolysaccharide. Colonies of χ1846 and χ1849 grown for 24 h on L agar + Dap at 37°C were replica plated on D agar plus 1% $CaCl_2$ (anhydrous weight) and incubated at 30°C for 24 h. The mucoid colonies of χ1846 were gently scraped off the plates and suspended in 5 ml of sterile deionized water. The rubbery texture and smaller volumes of the χ1849 replica plated colonies (relative to χ1846) required slightly more vigorous scraping. A greater number of plates were scrapped for χ1849 before a large enough suspension DAP-independent survivors was obtained. In each case care was taken not to include remnants of the agar surface. The scrapings were stored at 4°C for a few days before the volume of each was brought up to 30 ml with water. After vigorous vortexing, the scrapings were sedimented by centrifugation at 7000 rpm for 15 min in a SS-34 rotor at room temperature, the supernatant fraction collected, and the centrifugation was repeated. The top 10 ml of the final aqueous phase was drawn off and mixed with 30 ml of 95% ethanol and left to sit for 20 min. The insoluble carbohydrate fraction was collected and vacuum dried at 55°C. The residue from drying was hydrolized in 1N HCl for 90 min and subjected to ascending paper chromatography (Whatman No. 1) using a N-propanol, ethyl acetate and water solvent (7:1:2) at room temperature. The chromatogramy, after air drying, was sprayed with p-anisidine (1.6 g) - phtalic acid (1.66 g) dissolved in 95% ethanol (100 ml) and heated for developing.

RESULTS

Bacterial Strains. Table 8 lists the genotypes of the strains used in this study. χ1776 is an approved component of an EK2 host-vector system and was derived in several steps from the intermediates, χ1846 and χ1849. χ1846 and χ1849 are isogenic save for the presence of the Δ49[gal-uvrB] mutation of χ1849, a deletion which extends throughout the entire gal operon. χ2447 was proposed for certification as the bacterial component of EK2 host-lambda system and was independently derived from an entirely different K-12 subline (for complete genealogy see Chapter 1). χ1846, χ1849 and χ1776 possess the dapD8 and Δ29[bio-asd] mutations and therefore require DAP as a consequence of a defective (dapD) and deleted asparate semialdehyde dehydrogenase (asd) gene. The dapD8 mutation reverts spontaneously at approximately 5×10^{-7} and its gene product retains according to Bukhari and Taylor (5), $\leq$ 40% of its enzymatic activity. χ2447 possessed a similar double block in DAP biosynthesis, however, the responsible alleles were different from those contained in the former three strains. Extremely stable point mutations of the asd and dapD cistrons reverting spontaneously at 1.8×10^{-9} and 7.7×10^{-9}, respectively contributed to this strain's Dap$^-$ phenotype. Isogenic strains possessing the dapD8 or dapD22 alleles when washed, concentrated and plated on PA exhibited varying abilities to produce a hazy "growth" on the medium lacking DAP. The strains possessing the dapD8 mutation, plated as described above, produced a

noticeable haze, while the dapD22 sibling had an all but undetectable growth under these same conditions and it was inferred that the reduced growth of the dapD22 strains was the result of a further decrease in the enzymatic activity of the dapD gene product. The "leakiness" of the dapD22 mutation, however, was radically increased in the presence of NaCl (see Chapter 1). The asd-3 allele was similarly unable to produce a "leaky" growth when plated on PA.

Colony Formation of Dap⁻ Bacteria in Absence of DAP. Colonies equal in size to those of the control (PA + DAP) were recovered with 100% efficiency when χ1846 had been replica plated to PA and incubated at 37°C for 6-8 h. Macroscopically the Dap⁻ colonies were mucoid. Microscopically the colonies were composed of bacteria which had lost their normal bacillary structure and were instead composed of spheroplast-like variants. The spheroplast-like forms could be suspended in liquid media lacking an artifical osmotic stabilizer without any apparent deleterious effects and it was possible to microscopically demonstrate the reappearance of the normal rod morphology upon suspension and growth in L broth + Thd, DAP.

The mucoid nature of the colonies suggested a possible relationship between DAP-independent survival and exopolysaccharide secretion. Classically, the production of colanic acid was shown to possess temperature optimums below 37°C and consequently a "thermosensitivty" at 42°C (17). It is also known that mutations which interrupt the synthesis of uridine diphosphate-galactose-6-

phosphate (galU and/or galE) also abolish the production of colanic acid (17). χ1849 is devoid of a galactose epimerase activity and was constructed to test the relationship between exopolysaccharide production and DAP-independent survival. The data in Table 9 illustrate the relationship between exopolysaccharide synthesis and the appearance of colonies for χ1846 and χ1849 in the absence of DAP. The formation of colonies for χ1846 was temperature dependent whereas no growth of χ1849 was detected irrespective of the temperature of incubation. Similarly, χ1846 (but not χ1849) was able to form colonies on penicillin containing media by the induction of similar levels of exopolysaccharide. The secretion of an exopolysaccharide in the presence of penicillin was, however, slightly reduced in the medium containing DAP.

Contribution of Cations to the Survival of χ1846 and χ1849 in the Absence of DAP. One of the more unexpected aspects of the induction of exopolysaccharide synthesis (and consequentially the induction of the ability to withstand DAP starvation) either by DAP-less or penicillin inducement was the absolute dependence for the inclusion of salts in the media. More specifically, it was the cation component which was needed (Table 10). On a cation for a cation basis approximately 30 mM of Mg^{+2} of either $MgCl_2$ or $MgSO_4$ (0.3% $MgCl_2$ or 0.4% $MgSO_4$) elicited the same maximal colony size as did 86 mM of Na^+ and 70 mM of Na^+ added as NaCl and Na_2SO_4, respectively, however, it was not possible to show a similar quantitative relationship for the anionic portions of the salts as maximum

TABLE 9. Exopolysaccharide formation in the absence of DAP and/or in the presence of penicillin: temperature dependence

Strain	Temperature of incubation after replica plating[a]			Penicillin[b]	
	32C	37C	42C	DAP	No DAP
χ1846	+	+	-	+	+
χ1849	-	-	-	-	-

[a] Strains were pregrown at 37°C on PA + DAP containing 0.8% NaCl for 24 h prior to replica plating. After replica plating the plates (PA containing 0.8% NaCl) were incubated at the temperature shown above. The presence (+) or absence (-) of exopolysaccharide was scored 12 h later.

[b] The same procedure described above was followed except that the PA + NaCl ± DAP plates contained 100 U penicillin/ml (potassium salt) and were incubated at 37°C.

TABLE 10. Effect of various salts on the production of exopolysaccharide in χ1846 incubated in the absence of DAP

Salt	% Salt (v/v)[b]						
	0	0.1	0.2	0.3	0.4	0.5	1.0
NaCl	-	-	+	+	++	+++	+++
KCl	-	-	-	-	+	+	++
Na_2SO_4	-	+	+	+	++	+++	+++
$MgCl_2$	-	+	++	+++	+++	+++	+++
$MgSO_4$	-	-	+	++	+++	+++	+++
$ZnSO_4$	-	-	-	-	-	-	-
$CaCl_2$	-	+	++	+++	+++	+++	+++

[a] Colonies of χ1846 pregrown for 24 h at 37°C (on PA + DAP were replica plated to D agar supplemented with the salts shown above and incubated at 37°C. Colonies were scored for the production of exopolysaccharide after 12 h. Symbols: (-) no exopolysaccharide produced, (+) low levels, (++) medium levels, (+++) maximum levels. Identical experiments done with χ1849 were negative for each salt

concentration tested.

[b] The approximate molar equivalent at 1% salt are: NaCl = 172 mM, KCl = 135 mM; Na_2SO_4 = 70 mM, $MgCl_2$ = 100 mM, $MgSO_4$ = 83 mM, $ZnSO_4$ = 54 mM and $CaCl_2$ = 90 mM. The media were adjusted to the salt concentrations listed above by the addition of an appropriate volume of a 10% stock. The 10% concentration was calculated from the anhydrous weights of the particular salt.

protection was produced at approximately 86 mM Cl^-, > 135 mM Cl^-, 60 mM Cl^-, and 56 mM Cl^- for NaCl, KCl, $MgCl_2$ and $CaCl_2$, respectively. It was also very evident that the protection of DAP-starved bacteria was not solely the result of reinstating a proper osmotic balance. Concentration of $MgCl_2$ and $CaCl_2$ which provided only 20% of the osmolarity of KCl provided the same maximum degree of DAP-less protection. A similar effect was seen for the other salts in Table 10.

The divalent cations exerted a more pronouced effect per mole than did the monovalent cations, an observation which is consistent with their increased valence and ability to undergo non-specific interactions with outer membrane components. The monovalent cations, Na^+ and K^+, also appeared to show a differential ability, barring any negative physiological effects, to protect bacteria growing in media devoid of DAP.

Chemical Analysis of Exopolysaccharide. The composition of the exopolysaccharide was assayed as described in the Materials and Methods. The four sugar components, galactose, glucose, glucuronic acid and fucose were present in the chromatographic analysis of χ1846. Only glucose of the four component sugars of colanic acid was present in the polysaccharide extract of χ1849.

Quantitative Assessment of the Effects of Salt on DAP-less Death. The replica plate method of quantitating the protection provided by the combined effects of exopolysaccharide and salt in DAP-starved

bacteria is limited by its reliance on a subjective assessment of the relative protection produced, and in addition introduces the variable of the solid agar surface. In Fig. 6, χ1846 and χ1849 were subjected to DAP deprivation in the presence and absence of NaCl (0.5%) under conditions conducive to the production of exopolysaccharide. It was immediately clear that χ1846's survival under these conditions was the direct consequence of NaCl. χ1846 survived as feebly as its non-capsule producing sibling, χ1849, when NaCl is lacking. Furthermore, χ1849 even in the presence of NaCl died more quickly than χ1846, surviving some 20-fold less well after 6 h. The addition of 110 mM sucrose to the salt deficient medium did not protect against DAP-less death for either χ1846 or χ1849 (data not shown). χ1846 and χ1849 were similarly (with reference to NaCl) affected by DAP-less incubation in the presence of Mg^{+2} or Ca^{+2}. In experiments in which NaCl, $MgCl_2$ and $CaCl_2$ were added to cultures of χ1846 and χ1849 1½ h after the initiation of DAP starvation, only a limited amount of protection was evident and most if not all of this was apparently the result of nonspecific interactions between the cations and cells (data not shown). The survival of χ1846 and χ1849 were quantitatively similar when cell wall synthesis was interrupted for 1½ h with penicillin prior to the addition of NaCl, $MgCl_2$, or $CaCl_2$ (data not shown). These observations are consistent with the hypothesis that all components essential to the protection of murein-less bacteria must be present relatively early in the induction process. χ1846 and χ1849 expired at similar rates in L broth + Thd which contains NaCl when their deaths were accelerated by

the synergistic effects of penicillin treatment and DAP starvation.

It is possible that the salts act on some receptor present on the Gal^+ parent, χ1846, but not its Gal^- derivative, χ1849, and that the production of an exopolysaccharide is independent of the survival noted for bacteria lacking a functional cell wall. It was predicted that the kinetics of survival for χ1846 in the absence of DAP and under conditions where exopolysaccharide synthesis was inhibited (i.e. 42°C) would be independent of the salt present if the cation portion interacts with the polysaccharide to provide an osmotic support. Alternatively the survival of χ1846 at 42°C would be dependent on exogenous NaCl if the salt exerted its effect through non-specific interactions with the cell surface rather than the exopolysaccharide. It is evident from the data in Fig. 7 that both components, polysaccharide and salt, were required for the potentiation of the survival phenomenon, for at 42°C the survival of χ1846 was virtually identical to that of χ1849 and its survival was independent of the ionic environment.

DAP-less Death. Fig. 8 illustrates the kinetics of survival of χ2447 when grown in L broth + Thd or ML + CAA, Bio, Thd. In L broth + Thd, χ2447 initially entered into a brief (0.5 h) stasis during which time the viability of the culture was only minimally affected by the absence of DAP from the media. Continued incubation was characterized by an expontential decrease in cell numbers in which the surviving population was reduced 10,000-fold. This last phase was

Figure 6. Effect of temperature and salt on the DAP-less death of χ1846 and χ1849. The procedures for growth and preparation of the χ1846 and χ1849 cultures are the same as described in Materials and Methods. The slight differences of starting titers were normalized to 2 x 10^7 cells/ml. χ1846 and χ1849 were suspended in L broth + Thd (●,▲) or D broth + Thd (○,△), respectively and incubated at 37°C.

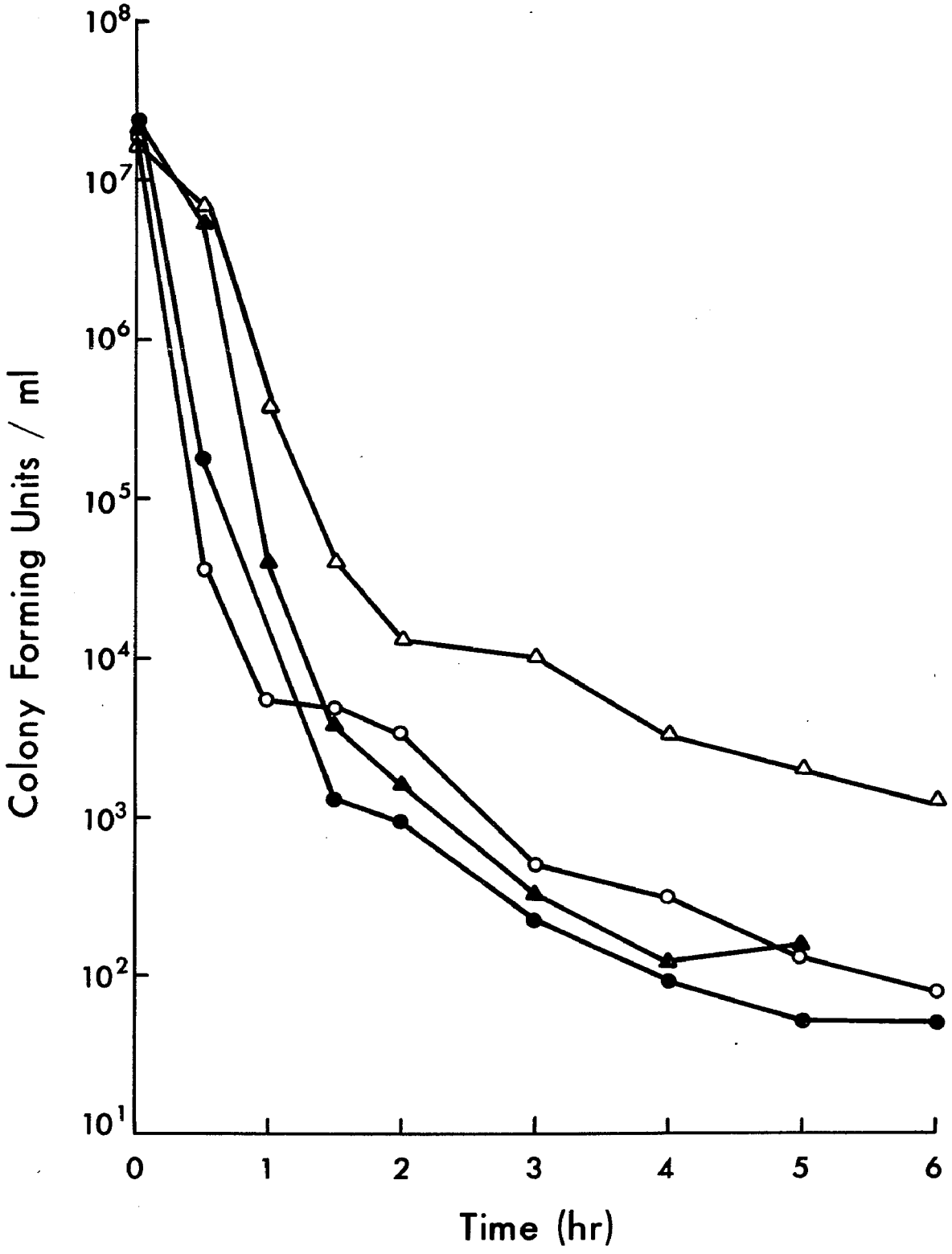
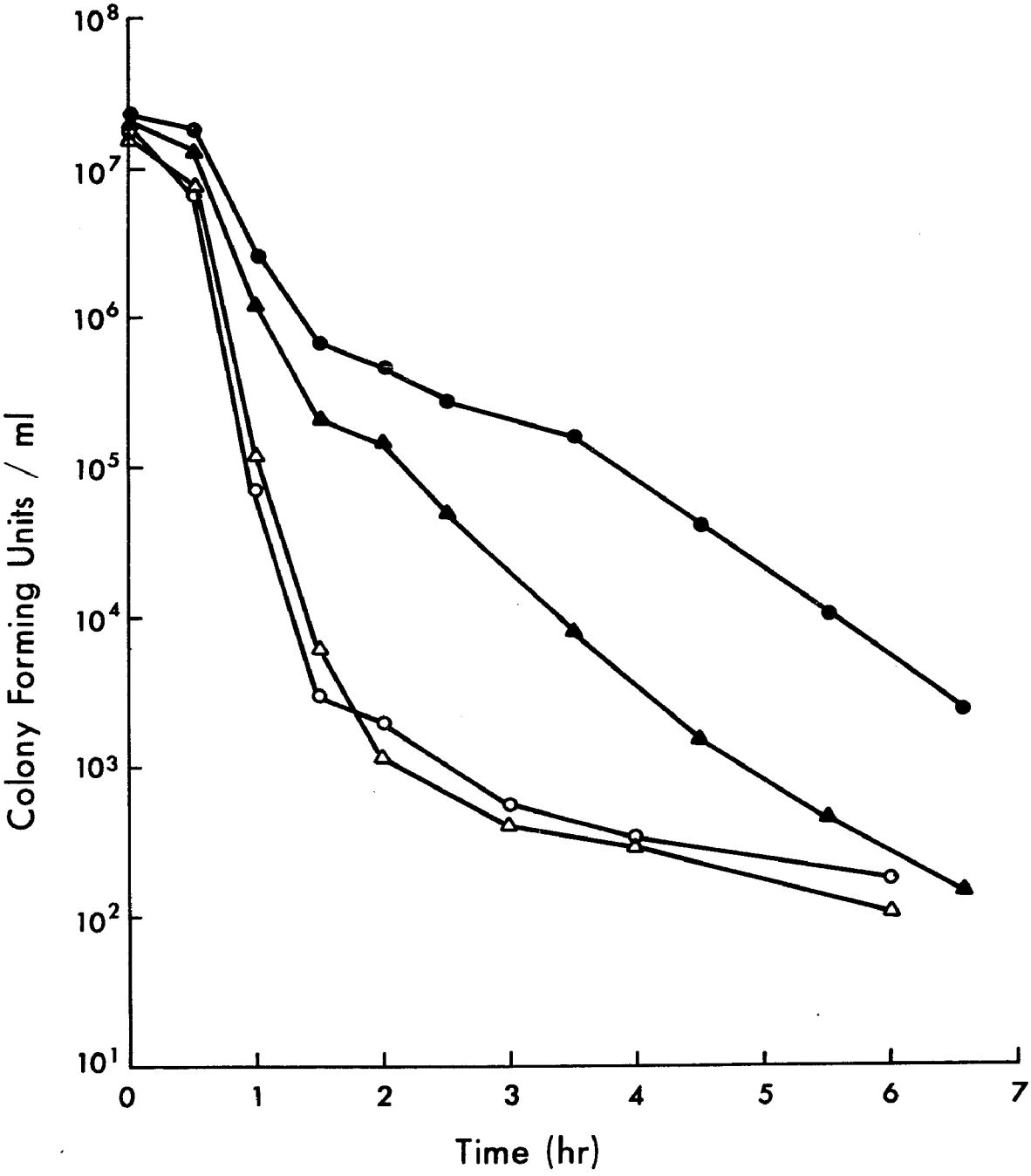

Colony Forming Units / ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
1
2
3
4
5
6
Time (hr)

Figure 7. Effect of temperature and salt on the DAP-less death of χ1846 and χ1849. The symbols and experiments were described in Fig. 6 except that the bacterial cells were incubated in the absence of DAP at 42°C. Symbols L broth + Thd (●,▲), D broth + Thd (o,Δ).


Colony Forming Units / ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
1
2
3
4
5
6
7
Time (hr)

followed by a gradual decrease in the rate of DAP-less death beginning at approximately 2 h of incubation and continued for up to 24 h and ultimately produced a 3 x 10^5-fold reduction in the starting titer. The first three phases of DAP-less death in ML + CAA, Bio, Thd (stasis, exponential death and decreasing rate of death) were not very different from those in L broth + Thd although the total extent of killing was reduced 3.5-fold. The last or fourth phase of DAP-less incubation was the regrowth that occurred in L broth + Thd and ML + CAA, Bio, Thd. The genetic properties of the surviving populations for χ2447 in L broth + Thd and ML + CAA + supplements were investigated. Colonies recovered after 8 and 48 h were tested for the retention of their Thy^-, Dap^- and Thr^- Met^- (asd) phenotypes. No Dap^+ or Asd^+ revertants were recovered among the survivors tested nor were Dap^+ survivors recovered either by direct plating of the 48 h culture on PA or by regrowing these same 48 h cultures in L broth + Thd DAP for 24 h prior to plating. The same clones which were previously tested for their Dap^+ phenotype after 8 and 48 h of DAP starvation were also scored as to their ability to grow on MA + Glc, Thr, Met, Bio, DAP, Thd. Some of the surviving bacteria from both L broth + Thd and MA + CAA had an additional auxotrophy (L broth 8 h, 2/42; 48 h, 7/43; MA + CAA 8 h, 6/50; 48 h, 17/49). Since DAP-less death is dependent on the growth of a bacterium the appearance of additional auxotrophy in the 8 and 48 h survivors might be thought to be a mechanism by which the bacteria avoid lysis. The latter explanation is not, however, compatible with the 3.5-fold greater killing observed in Fig. 6 for χ2447 grown in L broth + Thd vs. ML + CAA + supplements

Figure 8. DAP-less death of χ2447 in different media. In L broth + Thd (●) and ML + CAA, Bio, Thd (Δ).

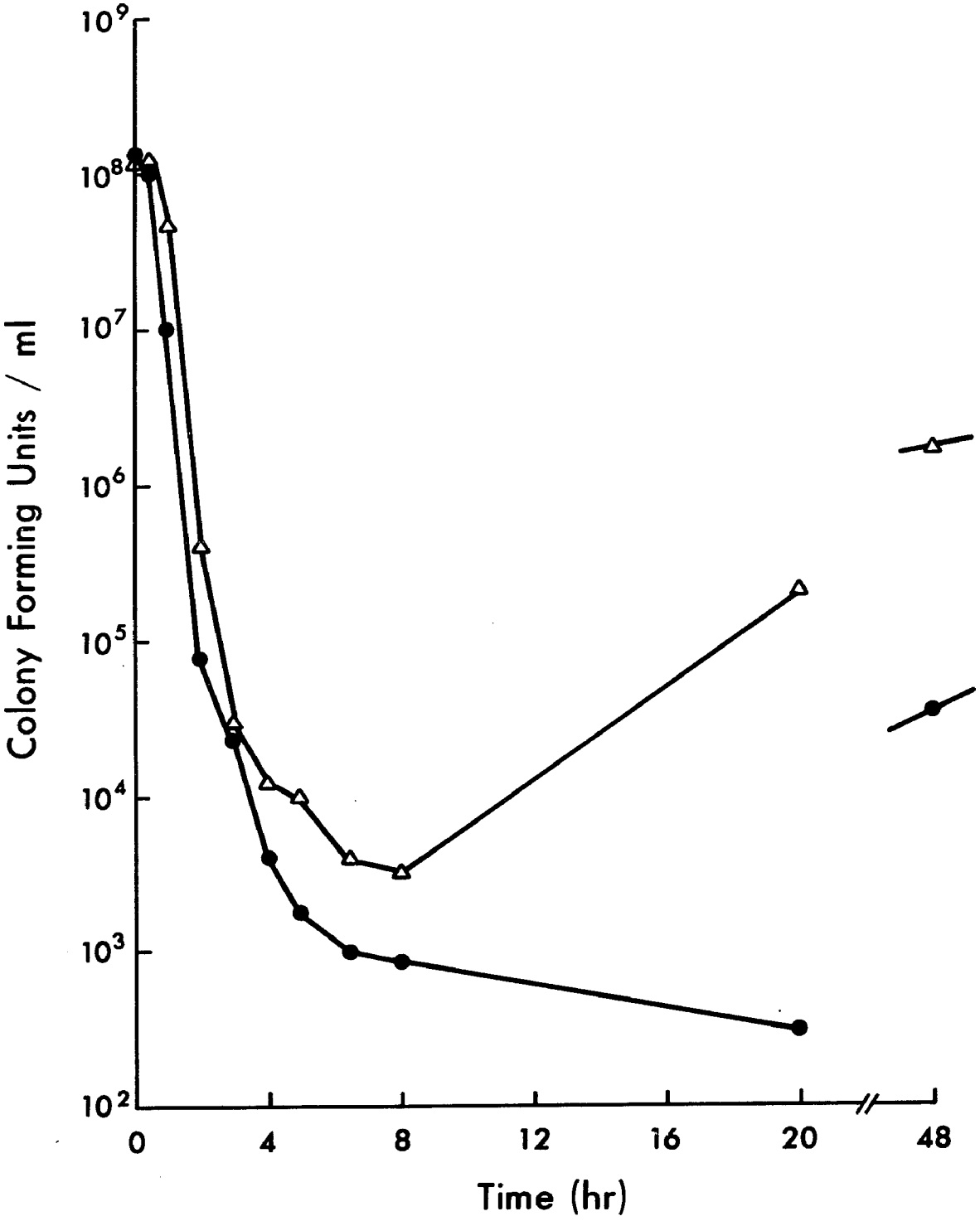
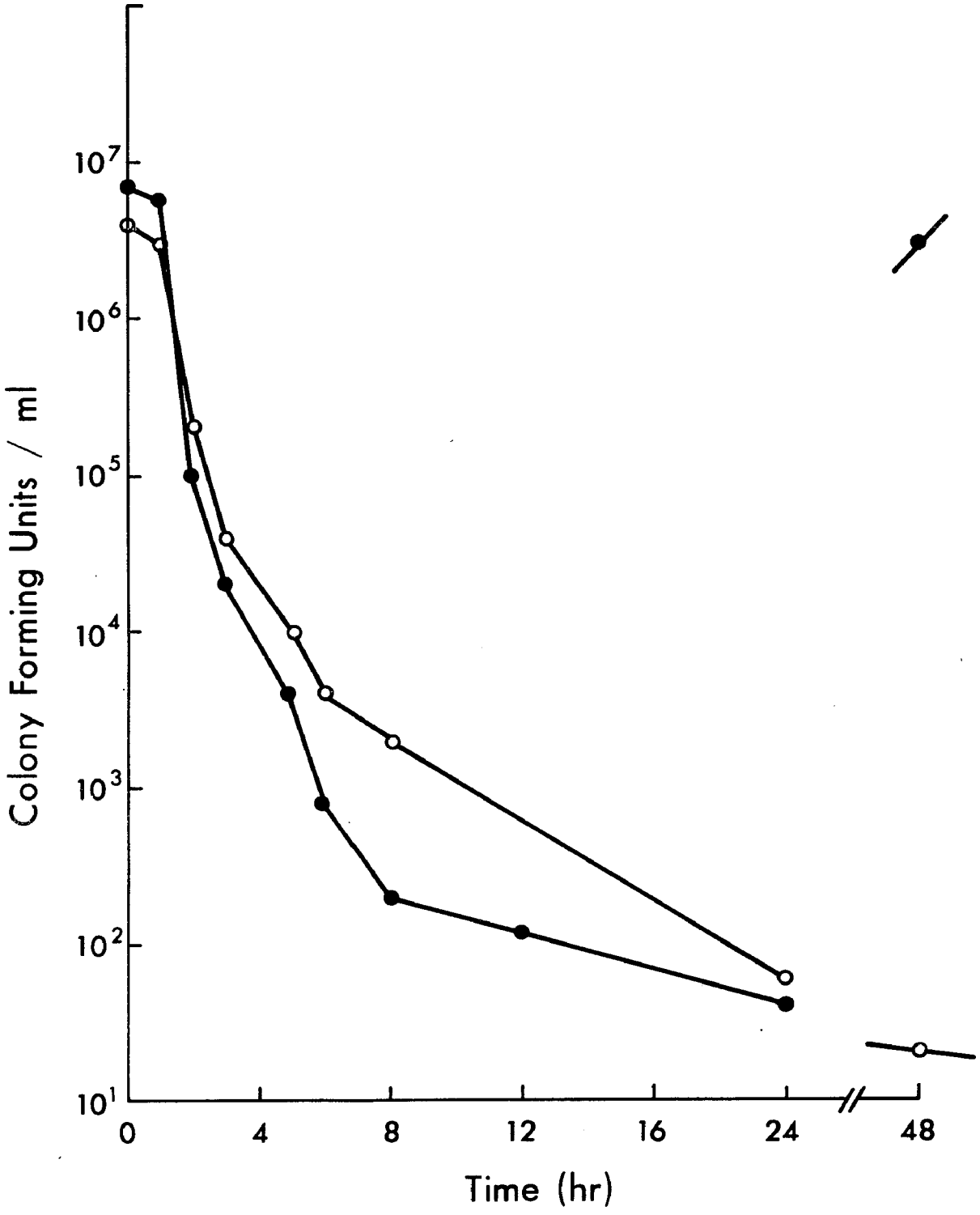

Colony Forming Units / ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
4
8
12
16
20
48
Time (hr)

Figure 9. Effect of NaCl on the regrowth of χ1776 incubated in the absence of DAP. At t=0 min a culture of χ1776 which had been growing exponentially in L broth + Thd, DAP was prepared as described in Materials and Methods and suspended in L broth + Thd (●) or D broth + Thd (o) at 37°C. Symbols: L broth + Thd, D broth + Thd.


Colony Forming Units / ml
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
4
8
12
16
24
48
Time (hr)

nor is it compatible with the observation that in nearly half the experiments no accumulation of additional auxotrophic bacteria was found. Morphologically the surviving colonies of χ2447 were generally smaller and by 3 or 4 days of incubation at 37°C achieved a maximum diameter of approximately 0.5 mm.

The regrowth phase was further characterized by an apparent 5-fold increase in generation time (relative to χ2447 grown in the presence of DAP). The contribution of 0.5% NaCl on the ultimate survival of DAP-deprived cultures of χ1776 is presented in Fig. 9. It is clearly evident that the omission of NaCl from the media relegated these populations to certain extinction, while cultures provided with salt are able to survive and even multiply in the absence of the "required" amino acid. It should be mentioned that χ2447 gave quantitatively similar results when tested in the above conditions (data not shown).

Rescue of DAP-less Death at Eight Hours. Fig. 10 illustrates the results of an experiment in which a sample of a culture of χ1776 which was undergoing DAP-less death was removed after 8 h of DAP-deprivation and inoculated into L broth + Thd, DAP and recultured. The "rejuvinated" population was again subjected to the same regime of DAP starvation and again a portion of the surviving fraction (at 8 h) was inoculated into L broth + Thd, DAP and the process repeated. Three characteristics are readily seen. First, the initial rates of DAP-less death did not appear to significantly differ

as the populations were repeatedly subjected to DAP-less incubation. Second, there was a corresponding decrease in the extent of killing of approximately 2 x 10^6-fold, 2 x 10^4-fold and 7 x 10^3-fold for the first, second and third passages, respectively. Finally all these cultures regrew in DAP-deficient media but to levels that did not seem to be qualitatively effected by the chronology of the passage.

Effects of Density on DAP-less Death. Bacteria which lyse because of DAP starvation release their cytoplasmic pools of this amino acid into the medium and through the action of autolysins and peptidases recycle the murein-stored complements of DAP to the surrounding environment. To study the effects of lysis on the regrowth phenomenon, four independent cultures of χ1776, two inoculated at 8 x 10^8 cells/ml and two at 5 x 10^6 cells/ml, were incubated in the absence of DAP (Fig. 11). The theoretical concentration of DAP incorporated into the cell walls of the two cultures suspended at 8 x 10^8 cells/ml is 1.1 μg DAP/ml while 5 x 10^6 cells/ml possess the equivalent of 7 μg DAP/ml. The greater than 100-fold difference in murein bound DAP did not result in a significant change in either the rates or the ultimate regrowth of the cultures (essentially identical results were obtained for χ2447, data not shown).

Figure 10. Kinetics of χ1776 subjected to repeated incubation in medium lacking DAP. χ1776 was subjected to DAP-less death in L broth + Thd as described in Materials and Methods except that after 8 h a sample was withdrawn and regrown in fresh L broth + Thd, DAP. The latter (regrown with DAP) culture was grown as before and subjected to the same conditions of DAP-less death and another sample was withdrawn and regrown after 8 h. Symbols: first DAP-less death (o), second (Δ) and third (X) DAP-less death. The arrows indicate the time at which the samples were withdrawn.

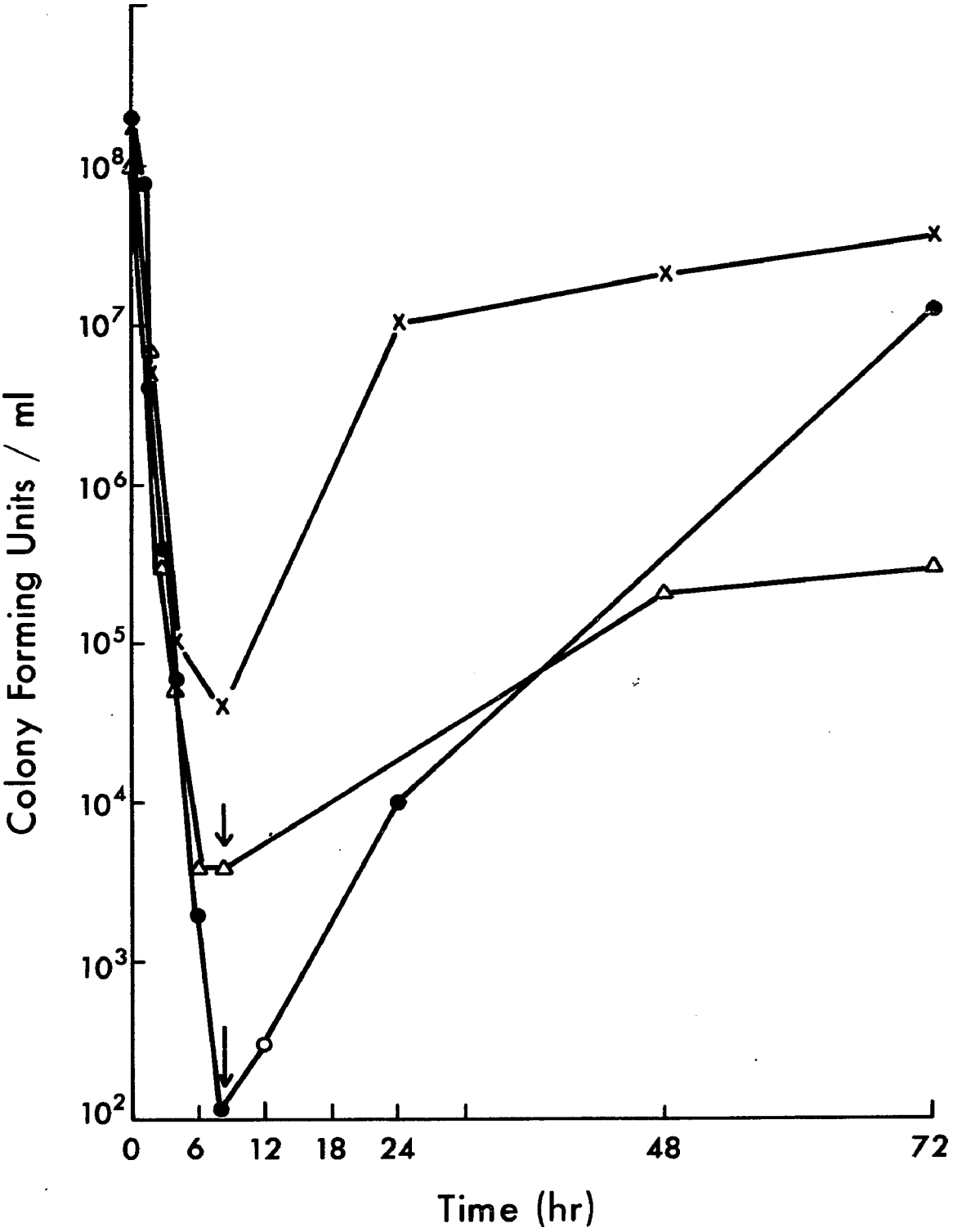
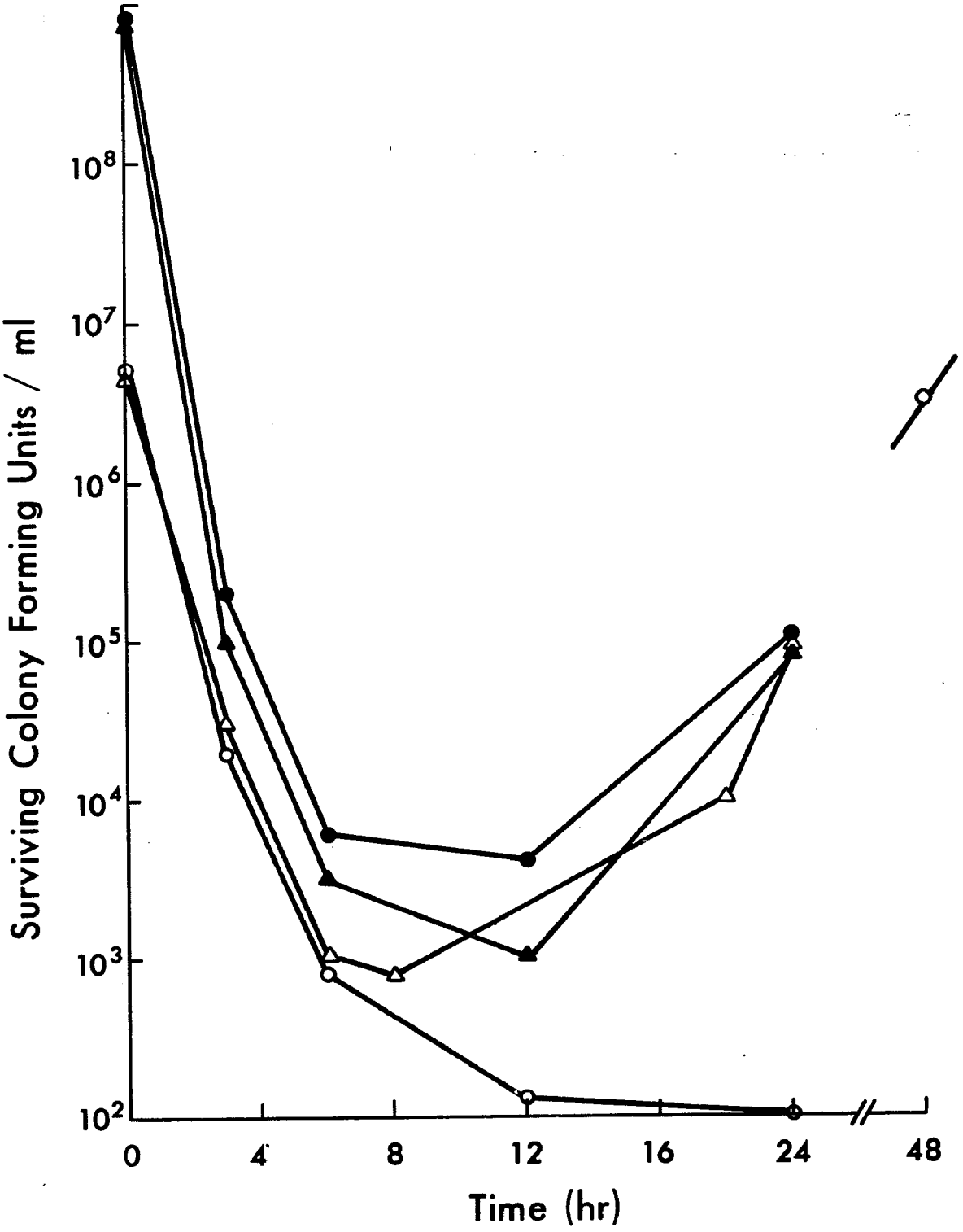

Colony Forming Units / ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
6
12
18
24
48
72
Time (hr)

Figure 11. Effect of the original cell density on DAP-less death of χ1776. Two exponentially growing cultures of χ1776 were prepared as described in the Materials and Methods before being suspended in fresh prewarmed L broth + Thd at each of the densities given above. Symbols: Suspended at 8×10^8 cells/ml, Experiment 1 (●); Suspended at 5×10^6 cells/ml, Experiment 1 (o); Experiment 2, suspended at 8×10^8 cells/ml (▲); and Experiment 2, suspended at 5×10^6 cells/ml (Δ).


Surviving Colony Forming Units / ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
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4
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16
24
48
Time (hr)

DISCUSSION

The process of secreting an exopolysaccharide to suppress the lack of a rigid cell layer in Dap⁻ *E. coli* was found to possess the following characteristics: (i) it was dependent upon certain temperature limits, (ii) the bacteria lost this ability by deletion of the *gal* operon, (iii) the protection of the wall-less bacteria was directly dependent on the availability of certain cations, and (iv) the response to secrete the capsular material appeared inducible by the osmotic stress that resulted from incubation in media lacking DAP and/or by treating the cell with the murein synthesis inhibitor, penicillin.

The galactose moiety of colanic acid is synthesized via a UDP-galactose intermediate and bacteria possessing mutations in the *galE* locus are unable to synthesize colanic acid even at permissive temperatures (17). The protection of bacteria undergoing DAP-less death is dependent on temperature and the presence of a functional *gal* operon. The coincidence of the above properties for the exopolysaccharide secreted in response to a DAP-less environment to those described previously for colanic acid encouraged the former's assignment as colanic acid. The latter supposition was further supported by qualitative chemical analysis of the exopolysaccharide material present in the surviving clones of χ1846. It is therefore attractive to infer that the exopolysaccharide described in this

report is colanic acid, however, the unimpeachable assignment of this material as colanic acid must wait quantitative and configurational analyses. It is interesting to speculate that the protection offered by the secreted polysaccharide is the consequence of: (i) the direct ionic interaction of divalent cations with the carboxyl groups of the glucuronic acid and ketal substituents, in such a manner as to form a stable polysaccharide-salt netting around the bacteria. Ionic interactions of this type were described for marine algae possessing capsules containing glucuronic acid. Interestingly the latter algae capsules possessed an affinity for Ca^{+2} and Mg^{+2} (23) and (ii) its affinity to concentrate and sequester both monovalent and divalent cations due to the highly hydrated nature of the exopolysaccharide in the immediate vicinity of the bacterium which relieves the osmotic tension on the cytoplasmic membrane. It is also apparent that the necessary structural components must be present relatively early since little protection was seen if salts were added as soon as 1½ h after the initiation of DAP-less death or if the rate of lysis was accelerated by the synergistic addition of penicillin.

Anderson (1) reported that the phosphate in Soreson buffer added at 0.2 M stimulated non-slime producing strains of Salmonella paratyphi B, E. coli and Arizona to produce an exopolysaccharide and later reported (2) that the Cl^- and SO_4^{-2} salts of Na^+, K^+, NH_4^+, and Mg^{+2} were able to elicit the same response at concentrations of 0.4 - 0.5 M (the variance between 0.2 M phosphate and 0.4 M Cl^-, SO_4^{-2} is probably the result of the inclusion of 0.85% NaCl in the Sorenson

buffered media). Anderson did not explore the contribution of the cationic portion of the individual salts to the induction of exopolysaccharide, although he was able to demonstrate that glycerophosphoric acid (adjusted to pH 7 with NH_4OH) was an equally active inducer of colanic acid secretion. However, the presence of 0.85% NaCl in the medium raises a doubt concerning his conclusion that metallic ions weren't necessary for the production of exopolysaccharide. Conflicting evidence was gathered for an exopolysaccharide secreting E. coli by Wilkinson et al. (26) who observed that the presence of excess carbohydrate in a medium limited in either N, P, or S caused an increase in polysaccharide production.

There are two apparent consequences of DAP-deprivation. First, the integrity of the cell wall is weakened and the existing osmotic imbalance between the cytoplasm and the surrounding environment can no longer be held in check by the mechanical presence of the murein layer. Secondly, the inability to complete the peptide portion of the growing UDP-N-acetyl muramic acid L-ala-D-glut precursor might result in an accumulation of essential regulatory intermediates, i.e. IPP.

Anderson et al. have reported data which suggest that an osmotic triggering of colanic acid synthesis is possible in E. coli and the penicillin induction of exopolysaccharide secretions can be interpreted as supporting the above (2). There is also data which suggest that the accumulation of the intermediate, IPP, is the

causative inducer of exopolysaccharide synthesis. There are reports which support the apparent correlation between the interruption of murein synthesis and the production of exopolysaccharide in E. coli (21,25). The author's own experience in the isolation of Dap$^-$ mutants has been that the majority of Dap$^-$ mutants isolated in exopolysaccharide-proficient strains are mucoid even in the presence of DAP. Sutherland (23) hypothesized that priorities in the usage of the IPP intermediate exist for murein, LPS and colanic acid production with murein production predominant over LPS synthesis and LPS synthesis over exopoloysaccaride secretion. In this context, the induction of colanic acid secretion in bacteria undergoing DAP-less death may be the direct consequence of IPP accumulation through a deficiency in available Park nucleotide production. The accumulation of the former intermediate provides the stimulation necessary for LPS and colanic acid biosynthesis. The hypothesis is attractive, however, it would seem naive to suggest that the regulation of exopolysaccharide production could be attributed solely to either of the above, especially in light of the apparent inductive properties of bacteriophage and nutritional limitation.

Bacteria undergoing DAP-less death pass through four distinct phases; a short period of relative statis, exponential death, a gradual decrease in the rate of death, and a slow but significant increase in cell numbers. The initial statis exhibited by the DAP-deprived bacteria was the consequence of the relatively large internal pools of DAP. Bacteria incubated under conditions

unfavorable for growth exhibited little loss in viability irrespective of the presence or absence of DAP. A progressively decreasing rate of death was observed for bacteria growing with correspondingly longer generation times (data not shown). A prior rate of the exponential death phase should be proportional to that fraction of the population which is actively growing. In the third phase, the gradual decrease in death was the apparent consequence of both physiological and mutational events. χ2447 which possessed virtually identical generation times in L broth + Thd and ML + CAA, Bio, Thd underwent essentially similar rates of exoponential death irrespective of whether DAP-less death was initiated in L broth + Thd or ML + CAA, Bio, Thd, but differed in total survival (see Chapter 1). The gradual increase in survival rate, however, was not solely explainable by the accumulation of auxotrophic mutations since a slow down was still evident in cases in which the survivors retain their original phenotypic properties. The above observations concerning the third phase of DAP-less death support the hypothesis that the phenomenon is physiological in nature. It is possible that the low concentration of DAP available to the bacteria stimulate the slow down of DAP-less death. The progressively smaller extents of killing following secondary and tertiary DAP-less incubation (Fig. 10) suggest that the process is not entirely independent of mutation.

The more intriguing aspect of DAP-less death is the fourth and final phase, i.e. paradoxic regrowth of Dap^- bacteria multiplying in the absence of DAP. This phase is, however, dependent on an ionic

environment. There are several possible explanations for this regrowth; (i) the remaining bacteria have adapted themselves to the efficient scavenging of the DAP released upon the lysis and degradation of other members of the bacterial population, (ii) the surviving fraction synthesizes an osmotically stable murein in an entirely unique fashion and (iii) the bacteria compensate for a weakened rigid layer by producing other cell wall components that offset the osmotic fragility of the DAP-deficient murein layer.

The first explanation predicts that if indeed the bacteria were able to increase the efficieny at which they bind and/or transport DAP across the cell wall, then it might be expected that both the rate as well as the final titers upon regrowth would be proportional to the original pool of DAP. Statistical fluctuations aside, there was no significant change in the generation time of those regrowing χ1776 and χ2447 cultures inoculated at densities ranging from 8 x 10^8 to 1 x 10^6 cells/ml (cultures suspended at concentrations higher than 1-2 x 10^9 cells/ml appear to initiate regrowth some 5 or 6 h earlier than above; data not shown). Conversely, the final densities reached by these bacteria are generally 10-100-fold less than the original starting titers. In several instances, for both χ1776 and χ2447, titers equal or slightly higher than the original starting densities were recorded after 72-96 h of incubation in L broth + Thd or ML + CAA, Bio, Thd (data not shown). The latter results of which demonstrated regrowth equal to the original starting density prior to DAP starvation is of particular interest since the

total DAP available in the cell walls of 5 x 10^6 cells/ml is estimated to be 7 μg DAP/ml and an extremely efficient system of scavenging would be necessary.

The second explanation is supported by reports of the successful substitution of structural analogues of DAP (3-hydroxy-diaminopimelic acid β-hydroxydiaminopimelic acid, γ-methyldiaminopimelic acid, lanthionine and cystathionine) into the murein of E. coli (6,19).

The final explanation also cannot be eliminated or fully supported at present. Certainly there is precedence for extraneous changes in the cell wall that can protect against DAP-less death (see this paper) and there is a report of a murein-deficient bacterium which retained its normal cell morphology (18). It can be imagined that a defective murein, i.e. without crosslinks between DAP and alanine but with an increased amount of the sugar backbone, might be able to survive if additional support were provided by the inner and outer membranes. For example, changes in the outer membrane which increase the sequestering of positively charged divalent cations might contribute the necessary stability needed to stabilize a murein layer deficient in the normal levels of peptide crosslinks by restoring the rigidity of the murein net. In this regard, the surviving bacteria produce irregularly shaped colonies on PA + DAP, a characteristic that is often associated with cell wall changes especially of the outer membrane (personal observation).

Finally, the physiological conditions of the culture media itself cannot be divorced from these discussions, for the phenomena described in the above models could be directly dependent on the presence of an unidentified "inducer". In the absence of such an "inducer" suppression of the DAP-deficiency may not be possible and would therefore explain the inability to recover Dap^+ variants. The assignment of the latter explanation to the recorded observations of regrowth seems the more likely interpretation, however, will require further testing.

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