Stability of Plasmid Sequences in an Acute Q-Fever Strain of *Coxiella burnetii*

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(Received 22 September 1987; revised 15 March 1988)

The rickettsial pathogen *Coxiella burnetii* undergoes a variation in which virulent isolates (phase 1) become avirulent (phase 2) after repeated passage in a non-immunologically competent host. Biochemically, this variation is associated with a lipopolysaccharide modification and possibly other factors. Genetically, the regions of DNA responsible for phase variation have not been identified. We have sought to determine whether the plasmid identified in acute disease isolates, QpH1, which represents approximately 5% of the coding capacity of this organism is involved in phase variation. Plasmids from phase 1 and phase 2 variants (designated QpH1 and QpH2, respectively) were compared by restriction endonuclease digestion and Southern blot hybridization to determine whether sequence changes in the phase 2 plasmid might account for changes in the virulence of phase 2 organisms compared with that of phase 1 cells. Using over 20 different restriction enzymes, no changes in DNA restriction fragment patterns were detected regardless of whether the phase change occurred during egg or tissue culture passage. The plasmid-specific mRNAs produced from metabolically active, purified cells were identical for each phase type. Using QpH1 or QpH2 DNA as a template, the mRNA produced by an *E. coli* extract was also identical. Finally, the proteins encoded by either plasmid in an *in vitro* transcription/translation reaction were identical. These data indicate that within the limits of our analysis, the plasmid DNA from *C. burnetii* phase variants is structurally and functionally the same and is therefore unlikely to be involved in phase variation.

INTRODUCTION

Coxiella burnetii, the aetiological agent of both acute Q fever and chronic endocarditis, is a rickettsial pathogen with no clearly defined virulence factors. The only phenotypic change of acute disease isolates with a known relationship to virulence is phase variation (Fiset, 1957; Stoker & Fiset, 1956). The virulent organisms isolated from natural infections are defined as phase 1. These organisms undergo a variation to phase 2 after repeated serial passage in a non-immunologically competent host such as embryonated eggs or tissue culture cells (Baca & Paretsky, 1983; Bobb & Downs, 1962).

Differences in surface properties between the virulent and avirulent phases (Fiset & Ormsbee, 1968; Krauss *et al.*, 1977) and in the carbohydrate composition of phase 1 and phase 2 antigens (Schramek & Brezina, 1979; Baca *et al.*, 1980) have led investigators to compare this phase variation with the smooth-to-rough transition of various enterobacteria. Demonstration of quantitative and qualitative differences in the sugar compositions of lipopolysaccharides (LPS) isolated from the two phases supports this comparison (Schramek & Mayer, 1982). Characterization of several separately generated phase variant clones demonstrated specific LPS chain alterations (Hackstadt *et al.*, 1985). Several biological changes have been reported to be associated with phase variation (Hoyer *et al.*, 1963; Kazar *et al.*, 1975; Kishimoto & Walker,

1976), leading to the suggestion that an LPS change is not the only biochemical modification accompanying phase variation. Indeed, one report has identified a change in protein composition that accompanies phase variation (Williams & Stewart, 1984). No matter which change(s) result from growth of natural isolates without the pressure of immunological selection, they are likely to be mediated by some modification of DNA sequences, since they appear to be permanent.

One modification has been identified using *Hae*III restriction enzyme digestion analysis of DNA from phase 1 and phase 2 variants of *C. burnetii* strain Nine Mile (O'Rourke *et al.*, 1985). The largest restriction fragment of phase 1 total DNA was absent in similarly digested phase 2 total DNA. However, a similar change was not observed when DNA preparations from other phase 1 and phase 2 *C. burnetii* strains were compared.

The plasmid found in *C. burnetii* is a discrete DNA element which could undergo sequence changes resulting in phase transition. Using two restriction enzymes (*Eco*RI and *Pst*I), initial characterization of the plasmid from strain Nine Mile did not reveal any difference between phase 1 and phase 2 plasmid DNA (Samuel *et al.*, 1983). However, a plasmid sequence change could play a role in phase variation even though it was not detected in these studies, especially because of the large plasmid size (36 kb). A report confirming the absence of differences in restriction endonuclease patterns between phase 1 and phase 2 plasmid DNA postulates that despite the similarity in their DNA, these plasmids may play a role in virulence (Vodkin *et al.*, 1986).

In this paper, we describe experiments that compare, in detail, the structure and function of plasmid DNA in phase 1 and phase 2 cells which we have designated QpH1 and QpH2, respectively. We used detailed restriction analysis, measured mRNA produced by *C. burnetii* cells and an *E. coli* lysate with QpH1 or QpH2 as templates, and compared the proteins encoded by QpH1 or QpH2. From these experiments we determined whether or not the plasmid DNAs of phase 1 and phase 2 variants of acute *C. burnetii* isolates are structurally and functionally indistinguishable.

METHODS

Propagation and purification of rickettsiae. *Coxiella burnetii* isolates used were as follows: Nine Mile RSA 493, clone 7, a plaque purified phase 1 isolate; Nine Mile RSA 439, clone 4, a plaque purified phase 2 isolate; and a culture of RSA 493 continuously propagated in L-929 cells and typed as phase 2 by complement block titration (kindly provided by O. Baca, University of New Mexico, USA). The first two isolates were propagated in embryonated hens' eggs and purified from host material by a series of differential centrifugations and sucrose gradients, as described by Williams *et al.* (1981) and modified by Hendrix & Mallavia (1984). *C. burnetii* were isolated from L-929 cells using previously described methods (O'Rourke *et al.*, 1985).

DNA preparation, restriction enzyme digestions and subcloning. Purified *C. burnetii* were lysed, and supercoiled plasmid DNA was separated from relaxed DNA that contains both non-supercoiled plasmid and chromosomal DNA on a caesium chloride/ethidium bromide gradient as described by Samuel *et al.* (1983). The plasmid DNA was digested with restriction enzymes according to the manufacturer's recommendations. Plasmid restriction fragments (*Eco*RI) were cloned into the *E. coli* plasmid vector pUC19 (Yanisch-Perron *et al.*, 1985) using methods described by Maniatis *et al.* (1982). Total DNA was isolated by a procedure that we have described previously (O'Rourke *et al.*, 1985).

Electrophoresis and detection of DNA and proteins. DNA samples were analysed electrophoretically in 0.9% agarose gels prepared with Tris/boric acid or Tris/acetic acid buffer (Maniatis *et al.*, 1982). DNA was visualized by staining with ethidium bromide. Proteins prepared in the transcription/translation reactions were separated on 4% (w/v) stacking and 12.5% (w/v) analytical polyacrylamide gels (Laemmli, 1970). Proteins labelled with [³⁵S]methionine were visualized by fluorography (Bonner & Laskey, 1974). Proteins labelled with ¹⁴C were used as *M_r* standards (New England Nuclear). The DNA-DNA hybridizations were done using the Southern blotting technique (Southern, 1975) with nick-translated DNA as a probe (Rigby *et al.*, 1977). The blots were washed with 1 × SSC (150 mM-NaCl, 15 mM-sodium citrate, pH 7.0) at 68 °C.

mRNA production. (i) *In vitro/in vivo* transcription. Messenger RNA was labelled in purified rickettsiae that were incubated in a buffer allowing them to become metabolically active (Hackstadt & Williams, 1981). This procedure was adapted from Corbin *et al.* (1982). Briefly, purified *C. burnetii* cells, stored frozen at -80 °C, were resuspended at a concentration of 3 mg dry wt ml⁻¹ in P-25 buffer (25 mM-K₂HPO₄, 15 mM-NaCl, 152.5 mM-KCl and 100 mM-glycine, pH 7.0). Cells were incubated at 37 °C for 1 h before RNA labelling was done. Pre-incubation at low pH to

metabolically activate the cells or permeabilizing the cells in 1% (v/v) toluene prior to labelling so that nucleotide transport was not required and did not significantly enhance the amount of RNA labelled. Therefore, cells were suspended at 3 mg dry wt ml⁻¹ in 100 µl reaction mixtures which contained the following: 20 mM-K₂HPO₄ (pH 7.4), 20 mM-MgCl₂, 200 mM-NaCl, 10 mM-β-mercaptoethanol, 200 µM-spermidine, 400 µM-ATP, 400 µM-GTP, 400 µM-CTP, 50–100 µCi (1.85–3.70 MBq) [α -³²P]UTP ml⁻¹ [specific activity 800 Ci mmol⁻¹ (29.6 TBq mmol⁻¹)] and 10 µg rifampicin ml⁻¹. The reaction mixtures were incubated at 37 °C for the time indicated. The RNA was extracted by adding an equal volume of 4% (w/v) SDS and heating the preparation at 100 °C for 10 min. After cooling to room temperature, NaCl was added to give a final concentration of 0.5 M, and the lysate was extracted twice with phenol/chloroform (1:1, v/v). Unincorporated [α -³²P]UTP was removed using a 5 ml column of Sephadex G-50 in 10 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA. Fractions of 200 µl each were isolated. This labelled material was used to probe Southern blots of plasmid and total DNA. Blots were pretreated and hybridized at 37 °C in a mixture of 55% (v/v) formamide, 4 × SSC, 0.4% SDS and 250 µg total *E. coli* RNA ml⁻¹. After 48 h hybridization, filters were washed at room temperature four times with 2 × SSC, once with 10 µg pancreatic RNAase ml⁻¹ in 2 × SSC, and twice with 2 × SSC containing 0.4% SDS.

(ii) *In vitro* transcription. Messenger RNA was labelled in a bacterial, DNA-directed, cell-free system using *C. burnetii* plasmid DNA templates (Yang *et al.*, 1980). The cell-free mixture was purchased from Amersham. The procedure used for mRNA labelling was a modification of the manufacturer's methods in which amino acid substrates were omitted from the *in vitro* reaction to prevent translation of message. The RNA was labelled by including [α -³²P]UTP as a substrate for mRNA production. This reaction was stopped by incubation with 2 µl DNAase (0.1 µg ml⁻¹) at 37 °C for 30 min. The reaction mixture was then extracted once with 2 vols 0.1% SDS, 10 mM-Tris/HCl, pH 8.0, 10 mM-EDTA, then extracted twice with phenol/chloroform (1:1, v/v). The RNA was precipitated with 300 mM-sodium acetate and 2 vols ethanol at -20 °C. The labelled mRNA was then used in hybridizations, as described previously.

Plasmid protein analysis. Transcription/translation. Proteins encoded by *C. burnetii* plasmid DNA were produced by a bacterial, DNA-directed, cell-free system. The system was prepared according to the method of Yang *et al.* (1980). The cell extract was used as directed by the manufacturer (Amersham). Labelled proteins were separated by SDS-PAGE and visualized by fluorography, as above.

RESULTS

Comparison of plasmid density and copy number in phase 1 and phase 2 variants of C. burnetii

C. burnetii isolates in phase 2 differ from those in phase 1 in a variety of surface properties, including agglutinability (Williams *et al.*, 1981). As a result, sucrose gradient centrifugation procedures designed to effect good separation of phase 1 organisms from host contaminants are less effective with phase 2 organisms. However, phase 2 organisms purified in this manner were readily lysed and their DNA was released by the procedure used with phase 1 *C. burnetii*. Plasmid DNA was isolated from phase 1 and phase 2 organisms using a caesium chloride ethidium bromide isopycnic centrifugation technique. The density of plasmid QpH2 from phase 2 rickettsiae, as determined by refractive index, was the same as the density of plasmid QpH1 from phase 1 organisms (1.615 ± 0.003 g ml⁻¹). The yield of plasmid DNA, compared to that for chromosomal DNA, on a mol: mol ratio, was used to calculate the minimum copy number for each plasmid. Repeated (> 3 ×) isolation of plasmid DNA from phase 1 or phase 2 *C. burnetii* gave a copy number of 3 ± 1 plasmids cell for both organisms.

Comparison of restriction enzyme fragments and maps

The purified plasmid DNA obtained from phase 1 and phase 2 *C. burnetii* was digested with different restriction enzymes, including those originally used to map QpH1 (Samuel *et al.*, 1983). The digests were electrophoresed in agarose. Fig. 1 shows the results of a series of comparative double restriction enzyme digestions of plasmids QpH1 and QpH2. No differences were detectable between the digests of the two plasmids. Furthermore, a comparative analysis of restriction fragments generated by digestion of these two plasmids with 20 additional enzymes detected no differences. The sum of restriction fragment sizes of each digest were averaged to obtain an approximate size of 36 kb (M_r 2.4×10^6) for each plasmid.

Since it is difficult to isolate sufficient quantities of rickettsial plasmid DNA to map additional restriction sites, *Eco*RI restriction fragments were subcloned into *E. coli* strain HB101

Fig. 1. Comparative restriction enzyme double digestions of plasmids QpH1 and QpH2. Plasmid DNA from phase 1 or phase 2 organisms was digested by the restriction enzymes (1 µg DNA was digested by 2 U enzyme at 37 °C for 2 h) and electrophoresed on a 0.9% agarose gel. Lanes contained the following digests: A and B, *Kpn*I and *Xba*I; C and D, *Bgl*II and *Kpn*I; E and F, *Sal*I and *Xba*I; G and N, λ-phage DNA digested with *Hind*III for *M*_{sp} markers; H and I, *Xba*I and *Bgl*II; J and K, *Sal*I and *Bgl*II; L and M, *Kpn*I and *Sal*I; O, *Sma*I. Lanes A, C, E, H, J, L and O contain DNA from phase 1 cells; lanes B, D, F, I, K and M contain DNA from phase 2 cells.

using the plasmid vector pUC19 (Yanisch-Perron *et al.*, 1985). Comparative restriction digest analyses, including three enzymes that recognize four base pair sequences of these subclones have revealed no differences (data not shown).

Phase 1 organisms of the *C. burnetii* Nine Mile isolate that were repeatedly passed (over 6–8 months) in mouse L-929 cells were used to determine whether the host environment or a partial shift in the phase character of the rickettsiae affected the stable maintenance of plasmid sequences. Isolation of quantities of plasmid DNA sufficient for a comparison as previously described required more rickettsiae than are commonly obtained by purification from tissue culture. Therefore using Southern blot hybridization (Southern, 1975), we compared the plasmid from the tissue culture grown cells with plasmids QpH1 and QpH2, which had been isolated from egg yolk culture. Total DNA from organisms was isolated, digested with restriction enzymes, and separated on agarose gels for blotting. Plasmid QpH1 was then used as a radiolabelled probe to detect homologous sequences. In Fig. 2 an autoradiograph of such an experiment is shown. DNA with homology to plasmid QpH1 sequences is present in lanes A, B, C (*Eco*RI digests) and G, H, I (*Bgl*II digests). Twelve different restriction enzyme digestion patterns of such DNA preparations were compared using the plasmid QpH1 probe. All showed identical plasmid fragments from both host environments. The absence of any detectable homologous sequences in the DNA extracted from host cells not infected with *C. burnetii* indicated that the plasmid sequences were *C. burnetii*-specific and were not derived from the cell or from contaminating organisms (Fig. 4, lanes D–F, J–L).

Fig. 2. Comparison of plasmid DNA from phase variants generated during repeated embryonated egg or tissue culture passage. A Southern blot was probed with nick-translated plasmid QpH1 and washed with $1 \times$ SSC at 68°C . The autoradiograph shows the probe homology with $1 \mu\text{g}$ DNA digested with a restriction enzyme and electrophoresed on an agarose gel. Lanes A–F are digested with *Eco*RI; G–L are digested with *Bgl*II. Lanes A and G, Nine Mile phase 1 total DNA; B and H, Nine Mile phase 2 (generated in egg culture) total DNA; C and I, Nine Mile phase 2 (generated in L-929 cells) total DNA; D and J, DNA isolated from yolk sac tissue of uninfected embryonated eggs; E and K, DNA isolated from uninfected chicken embryos; F and L, DNA isolated from uninfected L929 cells.

mRNA (in vitro/in vivo transcription) comparison

The restriction enzyme comparison of the plasmids of phase variants samples only a limited portion of their sequences. Even though no variation was seen, a change in sequences could exist, altering their *in vivo* function. Plasmid curing and transformation experiments have not been developed for *C. burnetii* to determine whether plasmid QpH1, when introduced into a plasmid-less phase 2 isolate, would convert this plasmid-less isolate to phase 1. Therefore, several alternative techniques were developed to compare the functions of plasmids QpH1 and QpH2.

One technique was adapted from a procedure described previously (Corbin *et al.*, 1982) to label mRNA produced by isolated *C. burnetii* in a metabolically active condition (Hackstadt & Williams, 1981). Purified *C. burnetii* do not replicate outside of a host phagolysosome, but when incubated in an appropriate buffer, they metabolize energy sources such as glutamate and proline and incorporate these substrates into a variety of macromolecules (Hendrix & Mallavia,

Fig. 3. *In vitro/in vivo* messenger RNA production by purified phase 1 or phase 2 organisms. Messenger RNA from *in vitro/in vivo* reactions (see Methods) was hybridized with restriction fragments of plasmid DNA separated on a 0.9% agarose gel. (a) is the agarose gel used to make the Southern blots shown in (b). P2 and P1 indicate which nitrocellulose transfer was hybridized with ^{32}P -labelled mRNA made by intact Nine Mile phase 2 (P2) cells or Nine Mile phase 1 (P1) cells. Lanes in both P2 and P1 are plasmid DNA digested with the indicated restriction enzyme: A and E, *HindIII*-digested λ -phage DNA (M_r markers); B, QpH1, *EcoRI*; C, QpH1, *BamHI*; D, QpRS, *EcoRI*. (b) is an autoradiograph of (a) hybridized and washed with $2 \times \text{SSC}$ at room temperature, including a $10 \mu\text{g RNAase ml}^{-1}$ digestion. Plasmid QpRS was isolated from one strain of *C. burnetii* associated with chronic endocarditis (Samuel *et al.*, 1985). It was included in these studies as a control to evaluate the mRNA production of shared sequences.

Fig. 4. Messenger RNA production from plasmids QpH1 or QpH2 using an *E. coli in vitro* transcription system. Southern blots of plasmid DNA identical to those in Fig. 3 were used to hybridize with mRNA probes. Lanes in both P1 and P2 contain plasmid DNA digested with the indicated restriction enzyme: A, QpH1, *Eco*RI; B, QpH1, *Bam*HI; C, QpRS, *Eco*RI. Lanes labelled P1 were hybridized using mRNA from plasmid QpH1 DNA; lanes labelled P2 were hybridized using mRNA from plasmid QpH2 DNA.

1984). Phase 1 or phase 2 *C. burnetii* were incubated in such a buffer containing four ribonucleotide triphosphates (UTP was α - 32 P-labelled). The labelled mRNA was used as a probe to detect plasmid sequences that were homologous to the labelled message produced (Fig. 3). Some plasmid restriction fragments hybridized with a relatively large amount of homologous mRNA and some restriction fragments hybridized with very little mRNA. These differences were used to measure the relative transcriptional activity between plasmids QpH1 and QpH2 in this or similar blots with different restriction digests. The only difference in the restriction enzyme digest and hybridization patterns was a high M_r , *Bam*HI-generated band (Fig. 3, lane C, P2). This band was due to partial digestion of DNA.

Fig. 5. Fluorogram of proteins encoded by plasmids QpH1 or QpH2 using an *E. coli* *in vitro* transcription/translation system. Proteins produced by each template were loaded with equal radioactivity and separated on a 12.5% SDS-PAGE gel with a 4% stacking gel and prepared for fluorography. M_r as indicated.

mRNA (in vitro transcription) comparison

The mRNA produced by plasmids QpH1 and QpH2 was compared with a system which measures the promoter activity of bacterial sequences in an *E. coli* extract. This technique was adapted from the cell-free bacterial transcription/translation (T/T) system (Yang *et al.*, 1980). Template DNA, plasmid QpH1 or QpH2, was incubated in this extract system, excluding amino acids and including four ribonucleotides ($[\alpha\text{-}^{32}\text{P}]\text{UTP}$). This labelled mRNA was used as a probe for Southern blots of restriction enzyme digests of plasmid DNA (Fig. 4) in a manner similar to the *in vitro/in vivo* experiments described above. These data show that although a few regions were not transcriptionally very active, homologous messages were produced that corresponded to most restriction fragments. These experiments also demonstrated that plasmids QpH1 and QpH2 showed the same promoter activity.

Transcription/translation protein production

The proteins encoded by plasmids QpH1 and QpH2 were compared using an *in vitro* transcription/translation extract. Template DNA was incubated in an *E. coli* extract and the proteins encoded by this DNA were labelled with [³⁵S]methionine and analysed by SDS-PAGE (Fig. 5). While minor differences in the proportional densities of some bands appear in individual gels, no consistent differences in banding patterns were observed.

DISCUSSION

The restriction enzyme profiles of plasmid DNA from cloned isolates of the Nine Mile strain of *C. burnetii* was not changed in organisms which had undergone phase variation. This was demonstrated by comparing restriction fragment patterns of plasmid DNA of *C. burnetii* organisms propagated in either egg or tissue culture host cells and by using a wide variety of restriction enzymes (~20). Because restriction enzyme analysis provides only an approximation of DNA stability, we also developed two techniques to compare the function of *C. burnetii* plasmids. The mRNA produced by metabolically active, isolated *C. burnetii* cells was labelled and used to detect the regions on plasmids QpH1 or QpH2 that were transcriptionally active. Labelled mRNA was also produced by *E. coli* cell extracts to determine which plasmid sequences contained functional promoters recognized by *E. coli* RNA polymerase. In RNA labelling assays, plasmids QpH1 and QpH2 appeared to have identical transcriptional activity. Finally, an *E. coli* extract system was used to produce labelled proteins coded for by plasmids QpH1 and QpH2. Both plasmids encoded for proteins of identical M_r . These data indicate that the plasmids from phase 1 and 2 organisms are structurally and functionally identical and are therefore unlikely to be involved in phase variation.

Confirmation that the plasmid does not function in phase variation requires consideration of additional factors. We measured mRNA production by hybridizing labelled message to plasmid DNA restriction fragments separated by electrophoresis. Transcripts in phase 1 cells derived from larger restriction fragments might be replaced by other mRNA species produced by phase 2 cells that hybridize to the same region. This change would not be identified by our experiments but might be identified by Northern analysis. We attempted to compensate for this problem by also comparing the proteins encoded by the plasmid from each phase. These analyses revealed no differences. However, our experiments do not eliminate the possibility that plasmid encoded products may have a regulatory effect on chromosomally encoded genes associated with phase variation.

The experiments described are significant for understanding the mechanisms of pathogenesis and intracellular survival by *C. burnetii* for several reasons. First, while virulence factors are carried by plasmid DNA in some bacteria (Elwell & Shipley, 1980), data presented here suggest that the QpH1/QpH2 plasmids do not encode the LPS biosynthetic genes involved in phase variation. Expression of the LPS biosynthetic genes is presumably modified during phase variation and is probably the factor involved in the loss of virulence that accompanies phase variation. Secondly, both phase 1 and phase 2 variants appear to replicate equally well in the phagolysosome of the host cell. However, surface or other alterations which lead to avirulence, resulting in the formation of phase 2 organisms, are selected for only by growth in a non-immunologically competent host (e.g. cell culture or embryonated eggs). Therefore, the emergence of a large portion of phase 2 organisms in any given population of *C. burnetii* is not likely to occur in nature. As a result, it seems more important to study virulence factors involved in natural infections, such as those which determine whether a particular isolate causes acute Q fever or chronic disease (e.g. endocarditis).

All acute disease isolates of *C. burnetii* have the same plasmid and previous studies have demonstrated that while the chronic strains do not contain the QpH1 plasmid, sequences very homologous to the majority of QpH1 are maintained in these strains (Samuel *et al.*, 1985). The maintenance of these plasmid sequences in all isolates of *C. burnetii* examined to date and over long-term passage in the laboratory implies that they are essential for survival of *C. burnetii*. It is possible that these genes provide this organism with the genetic ability to survive and grow in

the unique niche provided by the harsh environment of the phagolysosome. The plasmid may also encode or mediate expression of virulence factors that determine whether an isolate will cause acute or chronic disease. In order for this to be true, the virulence factor(s) involved in a particular disease pathology must either be coded for by the unique regions of DNA found on the plasmid of a particular isolate or must be inactivated by a mutational event. We have begun cloning and sequencing experiments to examine these possibilities.

Finally, the technique used in this study to compare mRNA and proteins produced by *C. burnetii* can be used to answer additional questions about this obligate intracellular parasite. For example, the mRNA produced by replicating rickettsiae in infected L-929 cells might be compared to the message produced *in vitro/in vivo* by the metabolically active but non-replicating rickettsiae described here. This may detect plasmid or genomic sequences necessary for *C. burnetii* replication.

This work was supported by Battelle, Pacific Northwest Laboratories under contract DE-AC06-76RLO-1831 and by Public Health Service grant AI20190 from the National Institute of Health.

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