Purification of Recombinant Human Alpha-2a Interferon Without Using Monoclonal Antibodies

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Abstract This report describes a high-level expression of human alpha-2a interferon (IFNα-2a) in *Escherichia coli* and its pilot scale purification by using a monoclonal antibody-independent chromatographic procedure that is based on anion-exchange, cation-exchange, hydrophobic interaction, and gel filtration. The recombinant *E. coli* produced much more IFNα-2a in a soluble form, when cultivated at low temperatures than at high-temperature fermentation. However, if the bacterial growth was taken into consideration, fermentation at 30℃ seemed optimal for the interferon production. By using our new protocol, we recovered approximately 160 mg of IFNα-2a with a specific activity of 3.59×10^8 IU/mg from 20 l of the broth. The gel permeation chromatographic and SDS-PAGE indicated that the interferon preparation was purified to homogeneity and was of the correctly folded fast-migrating monomer.

Key words: Recombinant *Escherichia coli*, cytoplasmic expression, fermentation temperature, human alpha-2 interferon

Rapid advances of recombinant DNA technology have made it possible to produce biomedically important substances in large amounts [1, 4, 7, 11, 15], one of which is human leukocyte interferon (IFNα-2). The most popular protocols for recovery of IFNα-2 synthesized in host bacteria, mostly in *Escherichia coli*, are based on the use of monoclonal antibodies [5, 17, 21, 22]. Although the antibody column has been proven to be a powerful tool for purifying crude interferon, a complete purification has never been achieved by one-step immunoaffinity chromatography. The main problem associated with this technique is contamination of immunoglobulin resulting from antibody shedding, that is, the release of antibody from the column through repeated use [8]. Furthermore, the antibody

column is unable to resolve various molecular species of the recombinant interferon which are formed in host cells during fermentation. Therefore, several other chromatographic steps are required to purify the native IFNα-2 preparation for therapeutic purpose. This implies that, regardless of using monoclonal antibodies, the purification of recombinant IFNα-2 may still rely on multi-step chromatographic procedures. In the present investigation, we devised a protocol for producing intact IFNα-2a on a pilot scale without using monoclonal antibodies.

MATERIALS AND METHODS

Materials
Escherichia coli MC1061, expression vector pBAD18, vesicular somatitis virus, and bovine kidney (MDBK) cell line were obtained from ATTC (Manassa, U.S.A.). Construction of a plasmid with human alpha-2a interferon cDNA and transformation of *E. coli* were performed essentially by following the standard manual of Sambrook *et al.* [14, 19]. International standard of human alpha-2 interferon was derived from NIH (Bethesda, U.S.A.).

Bacterial Culture and Protein Expression
The recombinant *E. coli* was grown in a preculture medium (12 g of trypton, 24 g of yeast extract, and 10 g of glycerol per liter, adjusted to pH 6.7 prior to autoclave) for 24 h at 30℃. The preculture (0.5 l) was then inoculated into a Nova Mitsuwa 50 FCD jar-fermenter (Mitsuwa, Osaka, Japan) with 20 l of a medium for the main cultivation (per liter, 20 g of trypton, 30 g of glycerol, 40 g of yeast extract, 9 g of K_2HPO_4, 6.3 g of KH_2PO_4, 5 g of Na_2HPO_4, 4.7 g of NaH_2PO_4, 20 g of NaCl, and 1 g of NH_4Cl, adjusted to pH 6.7), supplemented with filter-sterilized L-leucine (0.1%) and ampicillin (50 mg/l). The pH was automatically adjusted to 6.7 by adding NH_4OH or HCl during the fermentation process. Feeding of L-arabinose (1%) started

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3 h after inoculation, and fermentation was terminated after 11 h from the L-arabinose feeding. Under the fermentation conditions including the media compositions described above, pH (6.7), temperature (30°C), agitation (400 rpm), and aeration (1.5 vvm), we were able to increase the productivity level of soluble IFNα-2a up to 2×10^{10} IU/L-broth.

Purification Process

The following protocol was performed at 4°C.

Extraction. The biomass of broth was harvested by centrifugation, washed twice with a suspension buffer (10 mM Na-phosphate, 1 mM EDTA, and 0.5 mM PMSF, adjusted to pH 8.0), and resuspended in 10 l of the same buffer. The cells were disrupted with an M-110F Microfluidizer (MFIC, Newton, U.S.A.), and the homogenate was centrifuged (12,000 ×g) to retrieve a clear supernatant.

Anion-Exchange Chromatography (AEC). The supernatant was adjusted to pH 8.0 with 0.1 M NaOH and loaded onto a DEAE-Sepharose Fast-Flow column (10×20 cm), which had been equilibrated with 10 mM Na-phosphate (pH 8.0). The column was washed with the equilibration buffer, and proteins were eluted with 50 mM Na-acetate (pH 5.0) at a flow rate of 100 ml/min, and approximately 7 l of early fractions were collected.

Cation-Exchange Chromatography (CEC). The eluate from AEC was adjusted to pH 5.0 with 0.1 M acetic acid and loaded onto a SP-Sepharose Fast-Flow column (5×40 cm) which had been equilibrated with 0.1 M Na-acetate (pH 5.0). The column was thoroughly washed with the acetate buffer. Protein elution was carried out with a linear gradient of NaCl (0–0.4 M, pH 5.0) at a flow rate of 50 ml/min, and fractions with antiviral activity were pooled.

Hydrophobic Interaction Chromatography (HIC). Ammonium sulfate was added to the active pool (1.5 l) from CEC to a final concentration of 0.5 M, and the pH was adjusted to 6.5 with 0.1 M HCl. This was then loaded onto a Phenyl Sepharose 6 Fast-Flow column (5×10 cm), equilibrated with 50 mM Na-phosphate buffer (pH 6.5) containing 0.5 M ammonium sulfate. The column was washed with the same buffer until no protein was detected by 280 nm UV, and the elution was completed with a reverse linear gradient of ammonium sulfate (0.5–0 M, pH 6.5) at a flow rate of 10 ml/min.

Ultrafiltration (UF). The active fractions (300 ml) from the HIC column were ultrafiltrated by using a UF-membrane (Sartocon®-Micro VivaFlow 50, Sartorius, Goettingen, Germany) with a nominal molecular weight cut-off (NMWC) value of 30,000. The filtrate was concentrated to 15 ml on a UF-membrane of NMWC 10,000 and dialyzed against 50 mM Na-phosphate buffer (pH 6.5) with 50 mM NaCl.

Gel Permeation Chromatography (GPC). The dialyzate was subjected to gel filtration in two consecutive Sephacryl S-100 HR columns (2.6×100 cm each) which had been equilibrated with the dialysis buffer. Elution was carried out at a flow rate of 2.5 ml/min. The pooled fractions containing IFNα-2a was diafiltered and freeze-dried.

Biochemical Assays

Antiviral activity of IFNα-2a was determined by a cytopathic effect inhibition assay with vesicular stomatitis virus and MDBK cells, as described by Rubinstein et al. [18]. To assess the inclusion body-type interferon contained in the precipitates from cell extraction, it was solubilized in 7 M Guanidine-HCl and then renatured by the dilution process [9]. Interferon titers were expressed in a reference unit/ml calibrated against the reference standard for human alpha-2 interferon (Gxa 01-901-535, NIH, Bethesda, U.S.A.). Endotoxin in IFNα-2a preparations was quantitated by the limulus amebocyte lysate assay [10, 25] by using a commercially available assay kit (ENDO-LAL kit, Chromogenix, Moelndal, Sweden).

SDS-PAGE was performed according to Laemmli [12] by using commercially pre-cast 20% polyacrylamide gels. Prior to the electrophoresis, protein samples were SDS-treated at 100°C for 10 min in the presence or absence of 2-mercaptoethanol (10%) to denature under reducing and nonreducing conditions, respectively. Protein was measured by the Bradford method [2] using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

High-level expression of eukaryotic proteins in *E. coli* at high temperatures often gives rise to formation of insoluble inclusion body [13], whereas fermentation at lower temperatures tends to enhance the solubility of recombinant proteins in addition to the formation of correct disulfide bonds [16, 20]. Low temperatures have also been shown to stabilize IFNα-2 against proteolysis in two different Gram-negative bacteria, *E. coli* and *Methylophilus methylotrophus*, resulting in an increased accumulation of IFNα-2: for instance, when both organisms were grown at 37°C and 25°C, they possessed much greater interferon titers at the lower temperature [3]. In consistance, our preliminary results indicated that growth of the recombinant *E. coli* at lower temperatures gave greatly increased interferon titers per OD_{600} compared to the growth at higher temperatures (data not shown). However, fermentation at temperature as low as 25°C was apparently not feasible for commercial production of the recombinant interferon due to the severely limited bacteria growth. Based on the preliminary observations, a fermentation temperature of 30°C seemed optimal from the practical point of view. Expression data obtained at 30°C and 37°C are listed in Table 1. It is clear that, while the productivity level of the

Table 1. Effect of fermentation temperature on expression of IFNα-2a in *E. coli*.

Fermentation (°C)	Expression type	Activity* (×10⁹ IU/l)
37	Soluble	11.5±1.9
	Inclusion body	6.5±1.9
30	Soluble	15.5±1.8
	Inclusion body	1.6±0.8

*Data are means of triplicate measurements with SD.

total (soluble plus insoluble) IFNα-2a was almost the same, the 30°C fermentation provided much more IFNα-2a in a soluble form than the 37°C fermentation.

The overall scheme for preparation of IFNα-2a and progress of purification by the chromatographic steps are shown in Table 2 and Fig. 1, respectively. The pooled active fractions from the HIC column (the third chromatographic step) showed a high specific activity of human leukocyte interferon with purity greater than 80%. Through the ultrafiltration step, we were able to obtain highly purified (>95%) IFNα-2a with specific activity greater than the reported values (1.9–3.22×10⁸ IU/mg) of interferon products which were produced by major producers, such as Hoffmann-LaRoche [21], Schering-Plough [22], Interferon Science (US Patent 4,765,903), and Boehringer Ingelheim (US Patent 5,196,323). IFNα-2a preparation at this stage already appeared to meet CBER and CDER Guidances of FDA with respect to purity (>95%), specific activity (>1×10⁸ IU/mg), and endotoxin limit (0.10 EU/10,000 IU). Therefore, the last GPC step could be optional as a polishing step. However, this step would be necessary in cases when an interferon product essentially free of bacterial protein contaminants is desirable. If the GPC is to be chosen as a final step, then the previous ultrafiltration step may be omitted.

Purified about 180-fold with a yield of 17.2%, our final product showed a single peak on Sephacryl S-200 GPC with an estimated molecular mass of 18.5 kDa (data not shown) and a single band corresponding to 18.1 kDa on SDS-PAGE under reducing conditions strongly indicating that the protein was entirely of monomeric form. It was electrophoretically pure with no contaminating bands

Table 2. Purification of IFNα-2a expressed in *E. coli*.

Purification steps	Total protein (mg)	Total activity (×10¹⁰ IU)	Specific activity (×10⁶ IU/mg)
Extraction	166,440	32.6	2.0
AEC	16,864	17.8	10.6
CEC	2,070	10.8	52.2
HIC	289	8.7	301.0
UF	204	7.0	343.2
GPC	156	5.6	359.0

Data are a representative result obtained from 20 l of broth.
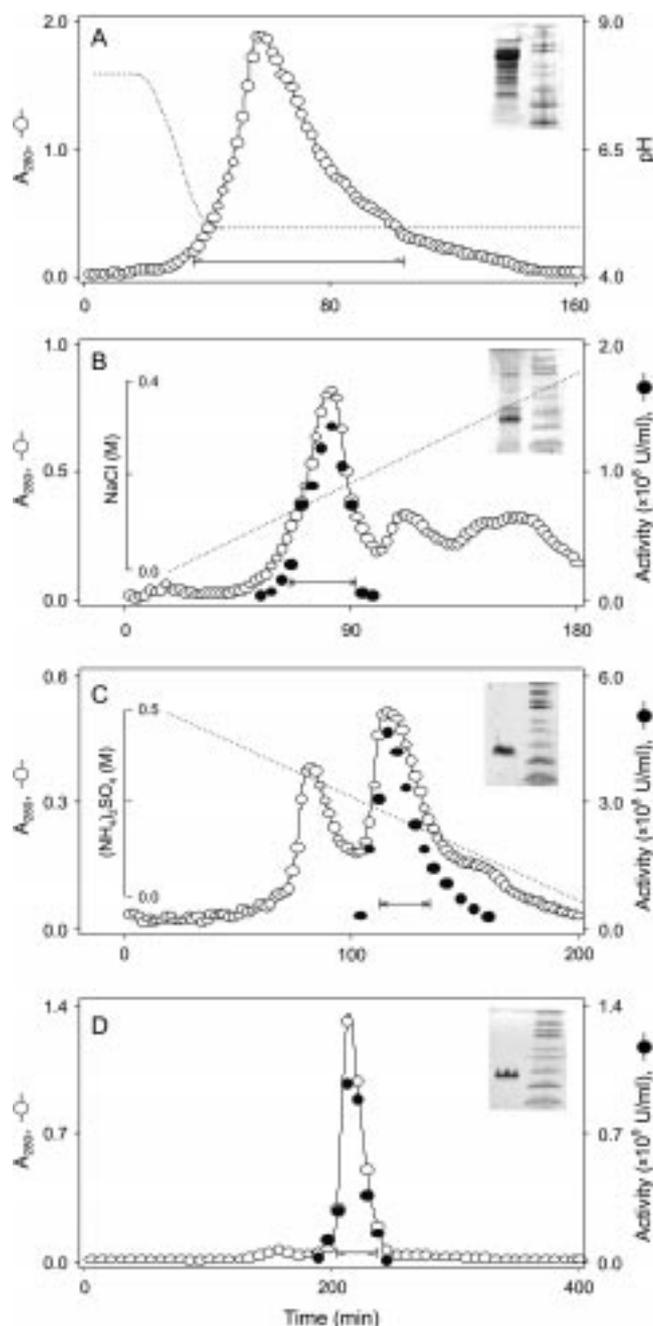

Fig. 1. Progress of IFNα-2a purification by chromatography. DEAE-Sepharose Fast-Flow (A), SP-Sepharose Fast-Flow (B), phenyl-Shepharose 6 Fast-Flow (C), and Sephacryl S-100 HR (D) columns were used in sequence. Double-headed arrows beneath the main peaks in the chromatograms indicate the fractions retrieved and pooled. Insets show the results of SDS-PAGE performed with the pooled active fractions under reducing conditions.

observed on the silver staining (Fig. 2A). The endotoxin level was estimated to be about 25 EU/mg-protein, i.e. 0.007 EU/10,000 IU, which is far below the allowed limit for clinical use. Two different molecular species of monomeric IFNα-2 have been known, which are referred to as fast-
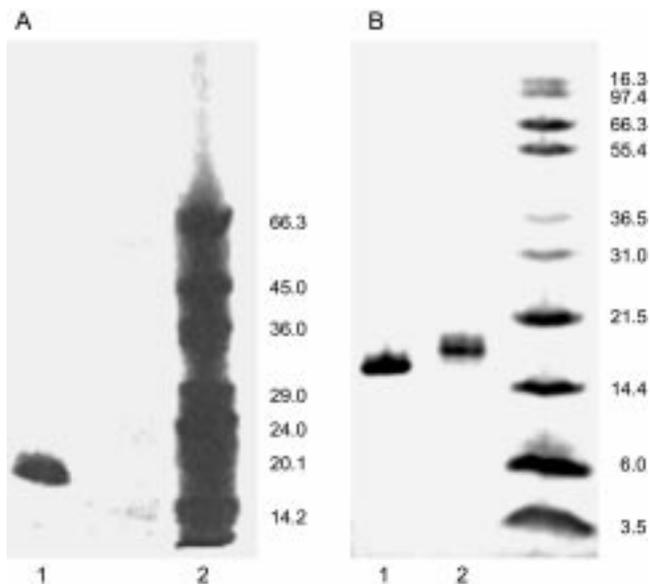
Fig. 2. SDS-PAGE of a final IFNα-2a preparation, visualized by silver staining (A) and Phastigel Blue R staining (B).
In panel A, lanes 1 and 2 are for IFNα-2a and blank under reducing conditions, respectively. In panel B, lanes 1 and 2 are for the same interferon under nonreducing and reducing conditions, respectively. Molecular weight markers in kDa are shown in each panel.

migrating monomer (FMM) and slow-migrating monomer (SMM), respectively, representing their relative mobility on SDS-PAGE under nonreducing conditions. FMM is the correctively folded and biologically active interferon that contains two intrachain disulfide bonds, one between Cys 1–Cys 98 and the other between Cys 29–Cys 138, while SMM only has one intact disulfide bond [24]. Interestingly, it has been shown that the relative mobility of SMM under nonreducing conditions is the same as under reducing conditions, while FMM migrates significantly faster under nonreducing conditions. Such electrophoretic property provides a basis for differentiating two monomeric forms of the recombinant interferon [6]. The SDS-PAGE profile (Fig. 2B) with different band positions responding to reducing and nonreducing conditions suggests that our IFNα-2a product is indeed of the FMM type.

Virtually all the known multi-step chromatographic procedures for a large-scale production of recombinant IFNα-2, including those of the above-mentioned major producers, employ a monoclonal antibody column as a critical purification step. In a sense, an exception may be a process described by Voss *et al.* [23], which is basically a four-step chromatographic procedure using silica-adsorption, phenyl-Sepharose, sulphopropyl resin, and DEAE-Sepharose columns in sequence. The feasibility of this process has been demonstrated in recovering periplasmically expressed IFNα-2c. The periplasmic expression results in a correctly folded native IFNα-2c that can be readily released from the periplasm by an acid/base treatment of

the cells [23]. However, the chromatographic procedure of Voss *et al.* has not yet been applied to whole cell extracts to purify cytoplasmically produced recombinant interferon, thus leaving a possibility that the method may be applicable only to samples containing smaller amounts of bacterial proteins with no other interferon species except for the native molecule.

To our knowledge, the present study is the first ever reported to describe a pilot-scale recovery of intact IFNα-2a from whole cells of recombinant *E. coli* by using a purification process that does not involve immunoaffinity chromatography. All the resins used for chromatographic columns in this investigation are not only readily available at reasonable prices, but are also stable enough to ensure a truly high longevity of gels, for repeated use. This may actually contribute to a significant share for lowering the production cost. In conclusion, our protocol appears as efficient as, but considerably less expensive than, monoclonal antibody-dependent methods.

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REFERENCES

1. Bae, K.-M., S.-K. Kim, I.-S. Kong, and H.-K. Jun. 2001. Purification and properties of cyclodextrin glucanotransferase synthesizing 2-*O*-α-D-glucopyranosyl L-ascorbic acid from *Paenibacillus* sp. JB-13. *J. Microbiol. Biotechnol.* **11:** 242– 250.

2. Bradford, M. A. 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye. *Anal. Biochem.* **72:** 248– 254.

3. Chesshyre, J. A. and A. R. Hipkiss. 1989. Low temperatures stabilize interferon α-2 against proteolysis on *Methylophilus methylotrophus* amd *Escherichia coli. Appl. Microbiol. Biotechnol.* **31:** 158– 162.

4. Chung, B. S., Y. T. Jeong, K. H. Chang, J.-S. Kim, and J. H. Kim. 2001. Effect of sodium butyrate on glycosylation of recombinant erythropoietin. *J. Microbiol. Biotechnol.* **11:** 1087– 1092.

5. Hochuli, E. 1986. Large-scale recovery of interferon α-2a synthesized in bacteria. *Chimia* **40:** 408– 412.

6. Honda, S., H. Sugino, K. Nishi, K. Nara, and A. Kakinuma. 1987. Purification of human leukocyte interferon A derived from *Escherichia coli*: A process to prevent the formation of its oligomer. *J. Bacteriol.* **5:** 39– 51.

7. Jang, E. K., K. H. Jang, I. Koh, I. H. Kim, S. H. Kim, S. A. Kang, C. H. Kim, S. D. Ha, and S. K. Rhee. 2002. Molecular characterization of the levansucrase gene from *Pseudomonas aurantiaca* S-4380 and its expression in *Escherichia coli. J. Microbiol. Biotechnol.* **12:** 603– 609.

8. Khan, F. R. and V. R. Rai. 1990. Large-scale production of recombinant proteins: Human leukocyte interferon, pp. 161–169. *In* S. Stein (ed.)*, Fundamentals of Protein Biotechnology*, Marcel Dekker, New York, U.S.A.

9. Kim, C. I., M. D. Kim, Y. C. Park, N. S. Han, and J. H. Seo. 2000. Refolding of *Bacillus macerans* cyclodextrin glucanotransferase expressed as inclusion bodies in recombinant *Escherichia coli*. *J. Microbiol. Biotechnol*. **10:** 632–637.

10. Kim, C. W., C. W. Christopher, and C. Rha. 1996. Removal of endotoxins and nucleic acids using submicron-sized polymeric particles. *J. Microbiol. Biotechnol*. **6:** 189–193.

11. Kim, S. Y., C. H. Lee, K. J. Kim, and Y. S. Kim. 2001. Expression of the functional interleukin-16 in *E. coli* and mammalian cell lines. *J. Microbiol. Biotechnol*. **11:** 234–241.

12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

13. Lee, I. Y., M. K. Kim, and S. B. Lee. 1992. Effect of induction temperature on the P_L promoter controlled production of recombinant human interleukin-2 in *Escherichia coli*. *J. Microbiol. Biotechnol*. **2:** 26–34

14. Lee, J., C. Ryou, and M. Kwon. 2001. Molecular cloning and expression of human dihydrolipoamide dehydrogenase-binding protein in *Escherichia coli*. *J. Microbiol. Biotechnol*. **11:** 592–597.

15. Mergulhão, F. J. M., G. A. Monteiro, A. G. Kelly, M. A. Taipa, and J. M. S. Cabral. 2000. Recombinant human proinsulin: A new approach in gene assembly and protein expression. *J. Microbiol. Biotechnol.* **10:** 690–693.

16. Mitraki, A., J. M. Belton, M. Desmadril, and J. M. Yon. 1987. Quasi-irreversibility in the unfolding-refolding transition of phosphoglycerate kinase induced by guanidine hydrochloride. *Eur. J. Biochem*. **163:** 29–34.

17. Rubinstein, M., S. Rubinstein, P. C. Familletti, R. S. Miller, A. A. Waldman, and S. Pestka. 1979. Human leukocyte interferon: Production, purification to homogeneity, and initial characterization. *Proc. Natl. Acad. Sci. USA* **76:** 640–644.

18. Rubinstein, S., P. C. Familetti, and S. Pestka. 1981. Convenient assay for interferons. *J. Virol.* **37:** 755–758.

19. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.* 2nd ed. Cold Spring Harbor Laboratory Press, New York, U.S.A.

20. Schein, C. H. and M. N. H. Noteborn. 1988. Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. *Biotechnology* **6:** 291–294.

21. Staehelin, T., D. S. Hobbs, H. F. Kung, C. Y. Lai, and S. Pestka. 1981. Purification and characterization of recombinant leukocyte interferon (IFLrA) with monoclonal antibodies. *J. Biol. Chem.* **256:** 9750–9754.

22. Trotta, P. P., H. V. Le, B. Sharma, and T. L. Nagabhushan. 1987. Isolation and purification of human alpha interferon, a recombinant DNA protein. *Developments in Industrial Microbiology* **27:** 53–64.

23. Voss, T., E. Falkner, H. Ahoron, E. Krystek, I. Maurer-Fogy, G. Bodo, and R. Hauptmann. 1994. Periplasmic expression of human interferon-α2c in *Escherichia coli* results in a correctly folded molecule. *Biochem. J.* **298:** 719–725.

24. Wetzel, A., P. D. Johnaton, and C. W. Czarniecki. 1983. Roles of the disulphide bonds in a human alpha interferon, pp. 101–112. *In* de Maeyer, F. and H. Schellekens (eds.), *The Biology of the Interferon System*, Elsevier, Amsterdam, The Netherlands.

25. Yin, E. T., C. Galanos, S. Kinsky, R. A. Bradshaw, S. Wessler, O. Luderitz, and M. Sarmiento. 1972. Picogram-sensitive assay for endotoxin: Gelation of *Limulus polyphemus* blood cell lysate induced by purified lipopolysaccharides and lipid A from Gram-negative bacteria. *Biochim. Biophys. Acta* **261:** 284–289.