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A New Procedure for Efficient Recovery of DNA, RNA, and Proteins from *Listeria* Cells by Rapid Lysis with a Recombinant Bacteriophage Endolysin

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A method for the rapid lysis of *Listeria* cells, employing a recombinant *Listeria* bacteriophage A118 lytic enzyme (PLY118), is described. The procedure can be used with all listerial species. It enables fast, efficient, and gentle recovery of DNA, RNA, or native cellular proteins from small-scale (2- to 5-ml) cultures. Moreover, this approach should be very useful in analytical detection and differentiation of *Listeria* strains when the release of native nucleic acids or proteins is required.

Listeria monocytogenes is one of six recognized species in the genus *Listeria* and is a facultative intracellular pathogen responsible for severe, food-borne infections in susceptible humans (reviewed in reference 8). *L. monocytogenes* and *Listeria ivanovii* are also pathogenic for some animals. Molecular research on these organisms requires methods for the extraction of nucleic acids and proteins, which should enable rapid but gentle release of these molecules from the rigid gram-positive cells. Flamm and co-authors (9) described a combination of lysozyme treatment with subsequent incubation in EDTA, protease, and sodium dodecyl sulfate (SDS) for extraction of chromosomal DNA. This procedure and various modifications derived therefrom (e.g., mutanolysin instead of lysozyme) have since been used by many workers (7, 10, 28). However, Lucas and Levin (19) reported considerable difficulty in releasing DNA from lysozyme-treated cells, unless high incubation temperatures (65°C) and high pH (9.0) were used. In our experience, we often obtained relatively low yields, which makes larger culture volumes necessary. The pulsed-field gel electrophoresis technique previously required at least 16-h incubations in a lysozyme-detergent solution (11, 20, 27) for the release of chromosomal DNA from listerial cells embedded in agarose plugs. Other methods used were lysis with guanidium thiocyanate (1, 22), a strong protein denaturing agent; or sonication (26), which inevitably leads to shearing of large nucleic acid molecules. Plasmids were generally extracted by a method adapted from that described for *Escherichia coli* (23), employing lysozyme and alkali treatment (13, 14). Incubation in hot phenol was described for RNA isolation (15, 21). Surface-associated proteins are readily removed with or without heating in the presence of detergents (2, 6), whereas *Listeria* cells remain intact during this treatment (24), therefore preventing the release of intracellular proteins. Besides the use of lysozyme, proteins could only be recovered by rigorous treatments such as prolonged sonication or repeated passage through a French press cell at 40,000 lb/in^2. However, these procedures are lengthy and relatively inefficient (17).

We have recently cloned three *Listeria* bacteriophage-encoded lysins (18), one of which (PLY118) could be overexpressed in *E. coli* to very high levels. The enzyme is extremely active on all listerial cells when added exogenously. Therefore, this study was conducted to simplify cell lysis in vitro by introducing a procedure based on the use of PLY118. The new technique greatly reduces the amount of time required and facilitates release and subsequent recovery of nucleic acids and proteins with high yields.

Preparation of lytic enzyme and cell suspensions and lysis procedure. *E. coli* JM109(DE3)(pPL118) carries the gene for PLY118 under the control of a strong, inducible promoter (18). Following overexpression of the enzyme, cells were disrupted by single passage through a French press cell or by sonication. Cleared cell extracts could then be fractionated by ion-exchange chromatography (Q-Sepharose; Pharmacia). Active fractions contained approximately 80% endolysin (18), which was found to be sufficiently pure for all downstream applications described here. The enzyme was stored in a suitable buffer (50 mM Tris Cl [pH 8.0], 20 mM NaCl, 0.1% Triton X-100) at −20°C.

L. monocytogenes WSLC 1001 (serovar 1/2c) and *L. ivanovii* WSLC 3009 (serovar 5) were from the Weihenstephan *Listeria* Collection. Cells were grown in tryptose broth (10-ml volumes, 30°C) to the end-log phase and harvested by centrifugation (2-ml portions; 15,000 × *g*, 60 s). Supernatants were completely removed, and pellets were quickly frozen in dry ice-ethanol or liquid nitrogen. For quantitative determination of lysin activity, cells were resuspended in 900 μl of buffer (20 mM Tris Cl, pH 8.0). Then, 100 μl of a PLY118 preparation was added. Figure 1 shows the observed decrease in absorbance, with the suspensions appearing completely clear after 8 to 10 min. One unit was defined as the amount of PLY118 necessary to decrease the A_{600} by 0.01/min at pH 8.0 and 30°C in a volume of 1 ml, using cells of *L. monocytogenes* WSLC 1001 as substrate. In this case, 100 μl of PLY118 solution contained approximately 10 U of enzyme activity. For preparation of nucleic acids and proteins, cells were resuspended in 50 μl of buffer and treated with 1 U (10 μl) of endolysin for approximately 5 to 8 min at room temperature until suspensions started to clear. The lysin can be used in combination with several common buffers in the range of pH 7.0 to 9.0 and attacks all listerial strains from the six species (18). It is also interesting that it is active at temperatures as low as 4°C, although at much lower rates.

Isolation of nucleic acids. Chromosomal DNA and total RNAs were essentially purified from the lysates according to standard methods (23), including deproteinization with phe-

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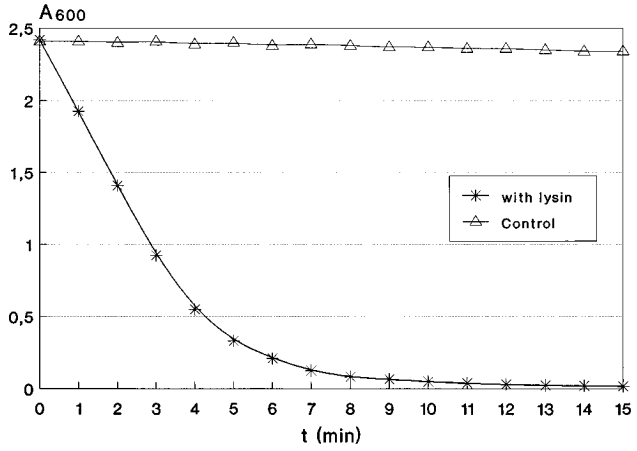

FIG. 1. Lysis of *L. monocytogenes* WSLC 1001 cells by cloned *Listeria* bacteriophage A118 endolysin PLY118.

nol-chloroform, ethanol precipitation, and digestion with either RNase-free DNase (Promega) or DNase-free RNase (United States Biochemical). Nucleic acids were resuspended in TE buffer (23) and quantified by photometric measurements. Yields ranged from 30 to 40 μg of total nucleic acids per 2 ml of culture, which is superior compared with the lysozyme or mutanolysin method, with which yields were generally 10 to 15 times lower (17). Qualitative analysis was performed on nondenaturing gels (Fig. 2) and clearly shows the integrity of chromosomal DNA as judged by the lack of "smear," which would indicate shearing or nuclease action. RNA isolated by this method has successfully been used in transcription mapping (18) and primer extension experiments (17). With a combination of amidase lysis and purification on ion-exchange resins in a spin column format (available from Qiagen or Scotlab), the selective isolation of listerial RNA from small-scale cultures is possible in approximately 30 min (data not shown).

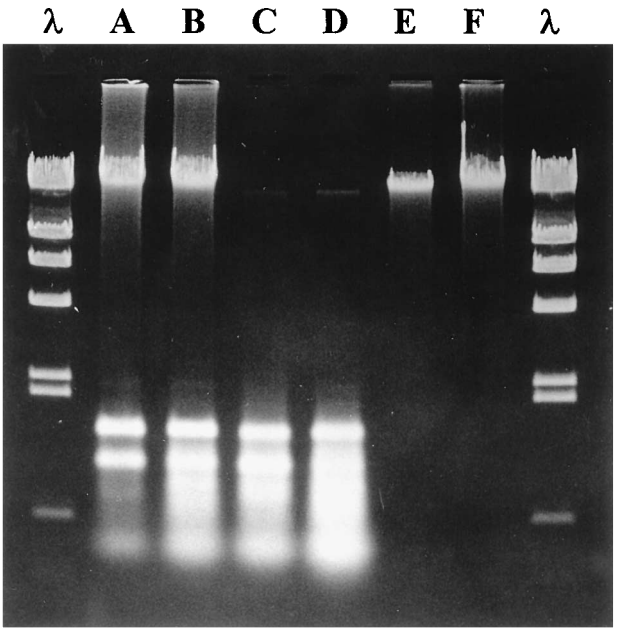

FIG. 2. Native nucleic acids prepared after rapid cell lysis with PLY118. Electrophoresis was performed in 0.7% agarose gels, and nucleic acids were stained with ethidium bromide. Lanes: λ, molecular size marker (lambda-*Hin*dIII); A and B, total nucleic acids extracted from *L. monocytogenes* 1001 and *L. ivanovii* 3009, respectively; C and D, purified total RNA from *L. monocytogenes* and *L. ivanovii*, respectively; E and F, purified chromosomal DNA from *L. monocytogenes* and *L. ivanovii*, respectively.

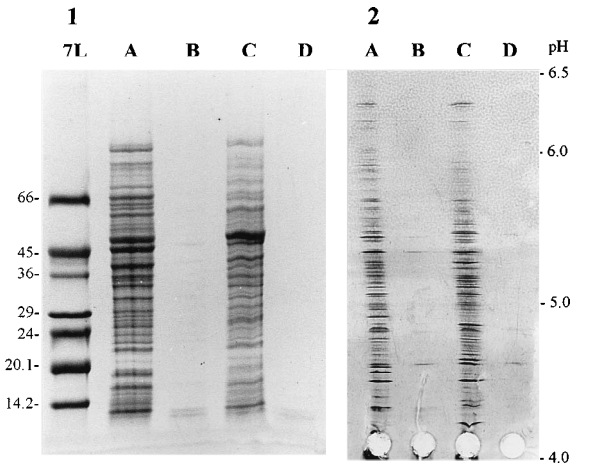

FIG. 3. Electropherograms of total cellular proteins released after endolysin treatment of listerial cells. (1) SDS-PAGE profiles on pore gradient (8 to 18% T) gels. Lanes: 7L, molecular size marker (indicated in kilodaltons); A and C, *L. monocytogenes* 1001 and *L. ivanovii* 3009 proteins, respectively; B and D, controls (without addition of lysin). (2) Protein patterns generated by isoelectric focusing in an immobilized, linear pH gradient (indicated on the right). Lanes are as in panel 1.

Preparation of total proteins. In order to reduce viscosity by removing nucleic acids, benzon nuclease (Benzonase; Merck) was added to the lysates (10 U per 50 μl); this was followed by incubation for 5 min at 30°C. Small samples (5 μl) were then removed and mixed with appropriate sample buffers before being analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing in immobilized pH gradients (pH 4 to 7) as described previously (16, 29). Untreated cell suspensions served as controls. SDS-PAGE gels were stained with Coomassie blue R-350 (Pharmacia), and isoelectric focusing gels were silver stained (5). The whole-cell protein profiles generated are shown in Fig. 3. Some cell wall-associated outer proteins are visible in the control lanes.

Conclusions. The method described here provides a simple and rapid procedure for lysis of *Listeria* cells which is superior to previously used techniques. Because of the short incubation times and ambient temperatures required for lytic activity, the inevitable nuclease or protease activities present in crude lysates were negligible; i.e., they did not interfere with the experiments conducted. This should be especially valuable for studies on listerial mRNA or shock-induced proteins, when speed is of the utmost importance.

Complete digestion with PLY118 should release even those proteins which are embedded in the thick cell wall structure of *Listeria* spp. and enable their isolation and study. Other interesting applications may include cell lysis prior to multilocus enzyme electrophoresis (4), treatment of samples subjected to analytical PCR for detection of *L. monocytogenes* (3, 12, 25), and cell lysis in agarose plugs prior to endonuclease digestion for pulsed-field gel electrophoresis analysis.

The concept of using recombinant phage lytic enzymes of high specificity and activity for research and biotechnological applications is presently being extended to other gram-positive genera (e.g., *Bacillus cereus* and *Staphylococcus aureus*) and will be reported in the near future.

REFERENCES

1. **Baloga, A. O., and S. K. Harlander.** 1991. Comparison of methods for discrimination between strains of *Listeria monocytogenes* from epidemiological surveys. Appl. Environ. Microbiol. **57:**2324–2331.
2. **Belyi, Y. F., I. S. Tartakovskii, and S. V. Prosorovskii.** 1992. Purification and characterization of cell wall proteins of *Listeria monocytogenes*. Med. Microbiol. Immunol. **181:**283–291.
3. **Bessesen, M. T., Q. Luo, H. A. Rotbart, M. J. Blaser, and R. T. Ellison III.** 1990. Detection of *Listeria monocytogenes* by using the polymerase chain reaction. Appl. Environ. Microbiol. **56:**2930–2932.
4. **Bibb, W. F., B. Schwartz, B. G. Gellin, B. D. Plikaytis, and R. E. Weaver.** 1989. Analysis of *Listeria monocytogenes* by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. Int. J. Food Microbiol. **8:**233–239.
5. **Blum, H., H. Beier, and H. J. Gross.** 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:**93–99.
6. **Bohne, J., Z. Sokolovic, and W. Goebel.** 1994. Transcriptional regulation of *prfA* and PrfA-regulated virulence genes in *Listeria monocytogenes*. Mol. Microbiol. **11:**1141–1150.
7. **Czajka, J., and C. A. Batt.** 1994. Verification of causal relationships between *Listeria monocytogenes* isolates implicated in food-borne outbreaks of listeriosis by randomly amplified polymorphic DNA patterns. J. Clin. Microbiol. **32:**1280–1287.
8. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
9. **Flamm, R. K., D. J. Hinrichs, and M. F. Thomashow.** 1984. Introduction of pAMβ1 into *Listeria monocytogenes* by conjugation and homology between native *L. monocytogenes* plasmids. Infect. Immun. **44:**157–161.
10. **Fliss, I., E. Emond, R. E. Simard, and S. Pandian.** 1991. A rapid and efficient method of lysis of *Listeria* and other gram-positive bacteria using mutanolysin. BioTechniques **11:**453–457.
11. **Howard, P. J., K. D. Harsono, and J. B. Luchansky.** 1992. Differentiation of *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, and *Listeria seeligeri* by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. **58:**709–712.
12. **Köhler, S., M. Leimeister-Wächter, T. Chakraborty, F. Lottspeich, and W. Goebel.** 1990. The gene coding for protein p60 of *Listeria monocytogenes* and its use as a specific probe for *Listeria monocytogenes*. Infect. Immun. **58:** 1943–1950.
13. **Kolstad, J., L. M. Rørvik, and P. E. Granum.** 1990. Characterization of plasmids from *Listeria* sp. Int. J. Food Microbiol. **12:**123–132.
14. **Lebrun, M., A. Audurier, and P. Cossart.** 1994. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are similar to *cadA* and *cadC* of *Staphylococcus aureus* and are induced by cadmium. J. Bacteriol. **176:**3040–3048.
15. **Leimeister-Wächter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty.** 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. Proc. Natl. Acad. Sci. USA **87:**8336–8340.
16. **Loessner, M. J., I. B. Krause, T. Henle, and S. Scherer.** 1994. Structural proteins and DNA characteristics of 14 *Listeria* typing bacteriophages. J. Gen. Virol. **75:**701–710.
17. **Loessner, M. J., and S. Scherer.** Unpublished data.
18. **Loessner, M. J., G. Wendlinger, and S. Scherer.** Submitted for publication.
19. **Lucas, R. D., and R. E. Levin.** 1989. Genetic transformation between strains of *Listeria monocytogenes*. Lett. Appl. Microbiol. **9:**215–218.
20. **Michel, E., and P. Cossart.** 1992. Physical map of the *Listeria monocytogenes* chromosome. J. Bacteriol. **174:**7098–7103.
21. **Oelmüller, U., N. Krüger, A. Steinbüchl, and C. G. Friedrich.** 1990. Isolation of procaryotic RNA and detection of specific mRNA with biotinylated probes. J. Microbiol. Methods **11:**73–84.
22. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
23. **Sambrook, J., T. Maniatis, and E. F. Fritsch.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Sokolovic, Z., J. Riedel, and W. Goebel.** 1993. Surface associated PrfA-regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. Mol. Microbiol. **8:**219–227.
25. **Starbuck, M. A. B., P. J. Hill, and G. S. A. B. Stewart.** 1992. Ultra sensitive detection of *Listeria monocytogenes* in milk by the polymerase chain reaction (PCR). Lett. Appl. Microbiol. **15:**248–252.
26. **Vines, A., M. W. Reeves, S. Hunter, and B. Swaminathan.** 1992. Restriction fragment length polymorphism in four virulence-associated genes of *Listeria monocytogenes*. Res. Microbiol. **143:**281–294.
27. **Wesley, I. V., and F. Ashton.** 1991. Restriction enzyme analysis of *Listeria monocytogenes* strains associated with food-borne epidemics. Appl. Environ. Microbiol. **57:**969–975.
28. **Wuenscher, M. D., S. Köhler, W. Goebel, and T. Chakraborty.** 1991. Gene disruption by plasmid integration in *Listeria monocytogenes*: insertional inactivation of the listeriolysin determinat *lisA*. Mol. Gen. Genet. **228:**177–182.
29. **Zink, R., and M. J. Loessner.** 1992. Classification of virulent and temperate bacteriophages of *Listeria* spp. on the basis of morphology and protein analysis. Appl. Environ. Microbiol. **58:**296–302.