JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1987, p. 2247–2251
0095-1137/87/112247-05$02.00/0

Vol. 25, No. 11

Listeria monocytogenes ATCC 35152 and NCTC 7973 Contain a Nonhemolytic, Nonvirulent Variant

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Received 5 June 1987/Accepted 14 August 1987

***Listeria monocytogenes* NCTC 7973 and this same strain deposited as ATCC 35152 contain two phenotypes: hemolytic virulent colonies and nonvirulent colonies that show no zones of hemolysis when streaked on heart infusion agar containing 5% rabbit blood. Results of examinations of these virulent and nonvirulent strains by investigators at the Centers for Disease Control, Atlanta, Ga., the Pasteur Institute, Paris, France, and the University of Würzburg, Federal Republic of Germany, support the conclusion that the avirulent strain is a nonhemolytic mutant of the virulent strain and that hemolysin is a virulence factor for *L. monocytogenes*.**

In November 1985, experiments were initiated in the Division of Bacterial Diseases, Centers for Disease Control (CDC), Atlanta, Ga., to study the physiological differences between species of *Listeria* as they have been defined on the basis of biochemical characteristics, DNA relatedness, and bacteriophage typing by Rocourt et al. (21–25). *Listeria monocytogenes* strains of different virulence for white mice were also examined.

The type strain *L. monocytogenes* ATCC 15313 was obtained from the American Type Culture Collection (ATCC). Based upon the request by Jones and Seeliger (14) for designation of a new type strain for *L. monocytogenes*, ATCC 35152 was also obtained since this strain, received by the ATCC from the National Collection of Type Cultures (NCTC) as NCTC 7973, possessed hemolytic and antigenic characteristics more common to *L. monocytogenes* than are those of the type strain, ATCC 15313 (NCTC 10357) (14). Because of interest in hemolysin as a possible virulence factor (2, 3, 10), we tested several blood-containing media for comparative hemolysis between the various strains (6, 9, 21).

Strain ATCC 35152 showed two types of colonies, one being markedly hemolytic when the agar contained 5% defibrinated rabbit blood in brain heart infusion agar (Difco Laboratories) or tryptose blood agar base (Difco) and the second showing no evident hemolysis after 24 to 48 hour of incubation at 37°C. These hemolytic and nonhemolytic colonies occurred in ratios approximating 3:1 to 2:1, respectively. Two hemolytic colonies and two nonhemolytic colonies were picked and restreaked onto rabbit blood agar; all of the strains showed only colonies having their original hemolytic activities, and first-generation stock cultures were prepared. Intraperitoneal injections in 3-week-old ICR (Institute of Cancer Research, National Cancer Institute, Bethesda, Md.) white female mice bred at the CDC showed that the nonhemolytic strains had 50% lethal doses (LD_{50}s) at least 100-fold greater than those of the hemolytic strains (Table 1). The cultures were restreaked, and a second generation of single colonies was picked and labeled KC1778+H1b, KC1778-H1b, KC1778+H2b, and KC1778-H2b. No hemolytic variants were observed when these colonies were picked or when they were restreaked; tests with mice showed differences in virulence similar to those observed with the first-generation cultures (Table 1).

The serological and biochemical characteristics of the first- and second-generation cultures were determined by a second laboratory (R.E.W.), and the original transfer made from ATCC 35152 was also reexamined. All four strains of the first- and second-generation colonies were of serotype 1/2a, and all were identical biochemically, including their sugar fermentations (6); hemolytic and nonhemolytic colonies were again observed when the original transfer of strain ATCC 35152 was restreaked onto blood agar. Also noted was slight hemolysis beneath the colonies of the nonhemolytic strains or very minimal zones of hemolysis around them, with continued incubation on rabbit blood agar plates. This type of hemolysis has been described previously by Seeliger (26). For reference only, these strains, showing only contact hemolysis as opposed to those giving zones of hemolysis, are referred to here as being nonhemolytic.

A second transfer of ATCC 35152 was examined by both the ATCC and CDC laboratories. This culture also contained both phenotypes. Since the original ATCC 35152 culture was obtained from the British National Collection of Type Cultures, a second culture of NCTC 7973 was obtained by the ATCC and was examined at the ATCC and the CDC. Both the hemolytic and nonhemolytic colonies were found by both laboratories. The second-generation strains described above were deposited with the ATCC and assigned the numbers given in Table 1; hemolysis by the hemolytic and nonhemolytic second-generation clones of ATCC 35152 is shown in Fig. 1.

In itself, the observation of hemolytic and nonhemolytic colonies in *Listeria* isolates is not new and had been reported several times many years ago (1, 9, 32). However, with the more recent delineation of species by Rocourt and her associates (21, 23, 24, 27, 29), hemolysin as a potential virulence factor can be more closely and unambiguously

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TABLE 1. Comparative LD_{50}s of *Listeria* strains

Species and CID strain no.	ATCC strain no.	Description	LD_{50}[a]	
			Expt 1[b]	Expt 2[c]
L. monocytogenes				
KC1778+H1b	43249	Hemolytic	2.0×10^5	3.0×10^5
KC1778+H2b	43251	Hemolytic	1.6×10^5	5.4×10^5
KC1778−H1b	43248	Nonhemolytic	$>2.4 \times 10^8$	$>3.7 \times 10^8$
KC1778−H2b	43250	Nonhemolytic	$>3.8 \times 10^8$	$>4.12 \times 10^6$
KC1775	15313^T	Type strain; nonhemolytic	$>6.0 \times 10^7$	$>2.0 \times 10^8$
F2380	43256	Isolated from Jalisco cheese; serovar 4b	2.0×10^4	1.7×10^4
F2381		Isolated from Jalisco cheese; serovar 4b		5.0×10^4
L. seeligeri KC1785	35967^T	Type strain; hemolytic	$>7.1 \times 10^6$	$>0.6 \times 10^6$
L. ivanovii KC1786	19119^T	Type strain; hemolytic	$>1.4 \times 10^8$	$>1.2 \times 10^9$

[a] Determined on day 5 after intraperitoneal injection in 3-week-old ICR white female mice. Numbers prefixed by the symbol > indicate no deaths at the highest dose tested. Inocula were grown for 24 h on tryptose blood agar base containing 0.1% glucose and 0.1% yeast extract. All inocula were adjusted to an A_{660} of 0.50 in 20-by-150-mm test tubes and diluted to 10^0 to 10^{-4}. CFU were determined at the time of inoculation.
[b] Experiment 1 was conducted with the first-generation cultures described in the text.
[c] Experiment 2 was conducted with second-generation cultures.

associated with *L. monocytogenes* than was previously possible. Also, identification of strains of *L. monocytogenes* relies heavily upon the presence or absence of hemolysins (14), since rhamnose-fermenting strains of *L. innocua* can only be differentiated from nonhemolytic *L. monocytogenes* by serotyping, phage typing, or DNA hybridization. Furthermore, within the last few years, results of four studies have been published that deal directly with the hemolysin as a virulence factor of *Listeria* sp. (8, 15, 18, 33). Of these, one (8) used strain NCTC 7973, and if it were not for the completeness of the investigations, interpretation of the data would be subject to criticism if the culture also contained the two phenotypes. In future studies, investigators need to be aware of the presence of these two phenotypes.

The observation that ATCC 35152 (and NCTC 7973) contains hemolytic and nonhemolytic colonies reemphasizes the strong need for a simpler phenotypic differentiation between *L. monocytogenes* and *L. innocua*. Also, the lower virulence of the nonhemolytic variant of ATCC 35152 strongly supports the conclusion that hemolysin is a virulence factor, as has been suggested by several publications (2, 3, 8, 10, 15, 16).

H. P. R. Seeliger, chairman of the International Subcommittee on the Taxonomy of *Listeria* and Related Bacteria, was notified of our findings. Examination of the NCTC 7973 culture in his collection by Seeliger and two laboratories at the CDC showed only hemolytic colonies when streaked on several blood agar media. The question was raised regarding the origin and nature of the nonhemolytic avirulent strains.

Through the auspices of H. P. R. Seeliger, the four ATCC strains derived from ATCC 35152 were distributed to various European laboratories for examination by phage typing and extracellular proteins and hemolysins. Similarly, these strains were distributed to several laboratories within the CDC. The tests performed, the results, and the comments on the hemolytic and nonhemolytic strains are as follows.

Serotyping (28) by R.E.W. showed that the four strains were of serotype 1/2a. Although of limited hemolytic ability, it is unlikely that an *L. innocua* strain is of serotype 1/2a.

Phage typing and serotyping (20–24) by J.R. revealed only three nonhemolytic strains of serovar 1/2a in 5,200 strains studied in her laboratory. The four strains were identical by typing with an official set of phages 1967, 2685, 4477, 575, 1652, 12029, 10, 19, 43, and 387 and with experimental *L. seeligeri* phages 3512 and 3794. All of the strains were identified as *L. monocytogenes*.

Extracellular protein characterization (18) by W.G. and S.K. showed that the sodium dodecyl sulfate-polyacrylamide gel electrophoresis-identified extracellular proteins of the four strains were identical to those of NCTC 7973 except for the absence of a ca. 58,000-M_r band in the nonhemolytic strains. This band was present in large amounts in filtrates of hemolytic strains ATCC 43249 and ATCC 43251 and the Mackaness strain (SLCC 5764) but was absent in M50 and M52 (nonhemolytic Tn*916* mutants). Similarly, this band was not observed in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses of whole-cell proteins of the nonhemolytic strains ATCC 43248 and ATCC 43250 (Fig. 2). In addition, the *L. innocua* strains surveyed had extracellular proteins markedly different from those of *L. monocytogenes*. The results state that the nonhemolytic strains 43248 and 43250 are mutants of the original strain.

Fermentation and biochemical analyses (6) by R.E.W. showed that all four strains were gram positive, motile, catalase positive, oxidase positive, methyl red positive, Voges-Proskauer positive, TS1 A/A, H_2S negative and milk negative (but with reduction); and sugar fermentations gave no gas, glucose A, xylose negative, mannitol negative, lactose A, sucrose A, maltose A, and rhamnose A. The negative hemolytic strains showed no CAMP reactions with *Staphylococcus aureus* or *Rhodococcus equi* (26).

Allozyme analyses (30, 31) by W.F.B. showed that all four strains had identical alanine dehydrogenase, alpha-naphthylpropionate esterase, isocitrate dehydrogenase, leucylglycylglycine peptidase, glucose-6-phosphate dehydrogenase, fumarase, phosphoglucose isomerase, indophenol oxidase, glutamate dehydrogenase, and phosphogluconate dehydrogenase.

Menaquinone analysis (5) by G.M.C. showed that all four strains contained MK5, MK6, and MK7 in the same quantitative distribution. The presence of these menaquinones agrees with the descriptions of *L. monocytogenes* and *L. innocua* (7).

Biochemistry and physiological analyses (4, 11, 17, 19) by L.P. and G.B.M. showed that growth was the same for all four strains under aerobic and anaerobic conditions in chemically defined broth, Casitone broth (Difco), sterile milk, and raw milk at 5 and 37°C. All four strains gave the same growth

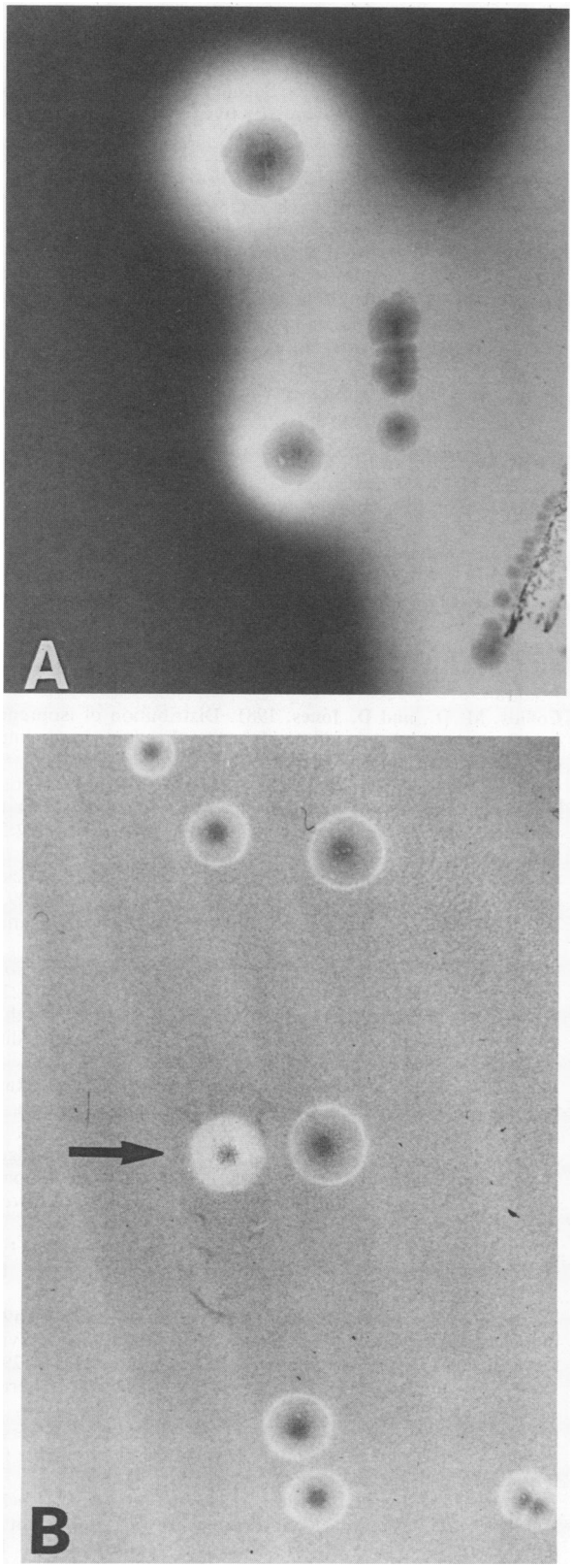

FIG. 1. Hemolysis by the virulent clone ATCC 43251 of *L. monocytogenes* ATCC 35152 (A) and by the avirulent clone ATCC 43250 (B). The center colony (arrow) was wiped off to show hemolysis beneath the colony. In contrast to the dark area of nonhemolyzed blood agar shown in panel A, the gray tone of panel B is due to the increased exposure time required to demonstrate the slight zones of hemolysis which did not completely penetrate the agar. Tryptose blood agar base plates containing 5% defibrinated rabbit blood were incubated for 4 days at 25°C. Magnification, ×414.

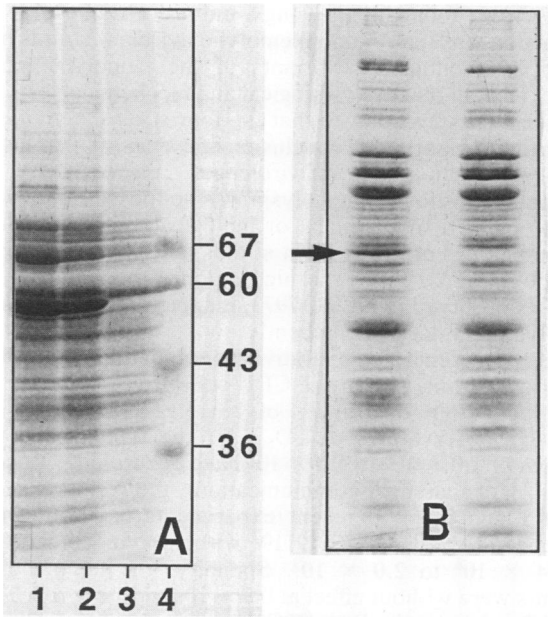

FIG. 2. Presence and absence of a protein (M_r, 58,000) in the extracellular proteins of the hemolytic strains *L. monocytogenes* SLCC 5764 and ATCC 43251 and its absence in the nonhemolytic strain ATCC 43250. (A) Extracellular proteins. Strains were grown overnight in brain heart infusion broth (Difco) at 37°C with shaking. Lanes: 1, SLCC 5764 (Mackaness); 2, ATCC 43251; 3, ATCC 43250; 4, the standards bovine serum albumin (M_r, 67,000), bovine catalase (M_r, 60,000), chicken egg ovalbumin (M_r, 43,000), and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (M_r, 36,000). (B) Total cellular proteins. Left lane, strain ATCC 43251 (the arrow indicates the 58,000-M_r protein). Right lane, strain ATCC 43250.

responses and/or cell yields when grown on measured amounts of glucose, galactose, lactose, rhamnose, and xylose. The four strains gave the same growth responses to LiCl, thallium acetate, acriflavin, cycloheximide, NaCl, nalidixic acid, KCNS, polymixin B, Tween 80, trypan blue, phenylethanol, glycine anhydride, coactin, and sodium azide. DNase and RNase, which were present in all four strains, did not differentiate the strains (12). Egg yolk lipase activity was found in all four strains (13, 17). Hemolysin-negative strains did not give CAMP reactions (25).

The hemolytic virulent and nonhemolytic avirulent strains were identical by all tests with the exception of the hemolysin reactions and virulence. Similarly, examination (W.G. and S.K.) of whole-cell and supernatant proteins of the four strains showed a protein with an M_r of 58,000 present in only the hemolytic virulent strains ATCC 43249 and ATCC 43251. This 58,000-M_r protein appeared identical to that observed in the Mackaness strain of *L. monocytogenes* SLCC 5764 (Fig. 2). The presence of this protein has been directly related to the virulence of this strain (15).

The major tests of serotyping and phage typing identified the cultures as *L. monocytogenes* serotype 1/2a; it is extremely unlikely that the nonhemolytic strains were *L. innocua* (J.R.). Because the hemolytic and nonhemolytic

strains were found in the single culture NCTC 7973, the conclusion was that the nonhemolytic avirulent strains originated as spontaneous variants of the hemolytic parent strain. That all other physiological and biochemical tests and allozyme analyses showed that the four strains were identical further supports this conclusion and suggests that loss of hemolysin results in loss of virulence.

Spontaneous loss of hemolysin was not observed, nor is it easily obtained by chemical or biological methods. Ten to twelve platings of the virulent strains failed to show nonhemolytic variants. From the high numbers of nonhemolytic colonies observed in NCTC 7973, it appears that this loss of hemolysin did not occur recently.

Table 1 presents a comparative determination of the LD_{50}s of various strains within the CDC collection; virulence was observed within the ranges of 2.0×10^4 to 5.0×10^5. Although observations of LD_{50}s for the type strain of *L. ivanovii* of 1.0×10^6 to 1.0×10^7 have occurred (J. Rocourt and H. Hof, personal communication), the results given in Table 1 and in more recent experiments have shown no virulence of strain ATCC 19119^T within the inoculum range of 1.4×10^8 to 2.0×10^9. Similarly, the nonhemolytic variants were without effect at 0.7×10^9 and 1.5×10^9 CFU. The LD_{50} for the virulent ATCC strains 43249 and 43251 in these later experiments was 3.0×10^5.

The observation of hemolysis rests upon the specific basal medium used and the source of erythrocytes. Thus, the type strain ATCC 15313^T, which showed no hemolysis of sheep erythrocytes in Trypticase soy agar (BBL Microbiology Systems), showed good hemolysis of rabbit erythrocytes in heart infusion agar. Nonhemolytic strains, however, may exhibit contact hemolysis beneath their colonies (Fig. 1). Direct comparative experiments were done at the CDC of the contact hemolysis of the avirulent strains ATCC 43248 and ATCC 43250 and that of six strains of *L. innocua* by using heart infusion agar with 5% defibrinated rabbit blood and Trypticase soy agar with 5% defibrinated sheep blood; incubations were made at 25 and 37°C. The results showed that some strains of *L. innocua* can give contact hemolysis of rabbit erythrocytes comparable to that observed with ATCC strains 43248 and 43250; thus, contact hemolysis does not appear to separate *L. innocua* from *L. monocytogenes* adequately. Also, such contact hemolysis does not appear to function on the basis of residual cellular hemolysin, since the analyses by S.K. and W.G. (Fig. 2) of whole-cell proteins suggest a total loss of the virulence-associated protein by strains ATCC 43248 and ATCC 43250. The nature of the contact hemolysis by these latter strains in unknown. Strain ATCC 15313^T showed classical zones of beta-hemolysis when plated on heart infusion agar with 5% rabbit blood.

As reported earlier by H. Hof (10), strain NCTC 10357^T (ATCC 15313^T), the type strain for *L. monocytogenes*, is avirulent. At concentrations of 6.0×10^7 to 2.0×10^8, however, mice inoculated with ATCC 15313 (Table 1) were lethargic and had ruffled fur for the first few days after infection, although they soon recovered. In tests with the avirulent variants ATCC 43248 and ATCC 43250, mice did not show signs of distress with inocula on the order of 10^8 to 10^9 CFU. H. Hof (10) described different degrees of virulence of *L. monocytogenes* in cultures characterized by the presence or absence of hemolysin and rough or smooth colony morphology; he emphasized that factors other than hemolysin may also play a significant role in the pathogenesis of *L. monocytogenes*. Nevertheless, the four strains described here (ATCC 43248, ATCC 43249, ATCC 43250, and ATCC 43251) appear to be new and useful research tools for studying hemolysin-associated virulence in *L. monocytogenes*.

We are indebted to H. P. R. Seeliger and H. Hof for their assistance, suggestions, and constructive criticisms during this investigation.

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