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Absence of Serotype-Specific Surface Antigen in Laboratory Variants of Epidemic-Associated *Listeria monocytogenes* Strains[▽]

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Variants that lacked reactivity with the serotype 4b-specific monoclonal antibody c74.22 and that lost susceptibility to certain *Listeria*- or serotype 4b-specific phages were identified in the course of genetic studies with serotype 4b *Listeria monocytogenes* strains H7550 and F2381L (epidemic clones I and II, respectively). Our findings suggest that such variants can become inadvertently established under laboratory conditions and suggest caution in work involving serotype 4b strains and genetic constructs thereof.

The majority of food-borne outbreaks of listeriosis have been associated with a small number of closely related strains ("epidemic clones") of serotype 4b. Epidemic clone I (ECI) has been involved in numerous outbreaks in Europe and North America (reviewed in reference 7). Epidemic clone II (ECII) was implicated in two multistate outbreaks of listeriosis in the United States, both of which involved contaminated ready-to-eat meat products (1, 2, 2a).

Serotype 4b strains harbor specific decorations in the teichoic acid (TA) of the cell wall that are required for reactivity with monoclonal antibodies (MAbs) such as c74.22, specific for strains of serotypes 4b, 4d, and 4e (8). At least two loci (*gtcA* and the *gltA*-*gltB* gene cassette) are required for reactivity of the bacteria with c74.22 and TA glycosylation (11, 15). Furthermore, *gtcA* mutants are deficient in invasion of several mammalian cell lines (14) and appear to be impaired for virulence in intragastric murine models of listeriosis (N. Faith, C. Czuprynski, Y. Cheng, and S. Kathariou, unpublished results). A population-level survey of epidemic-associated strains from three listeriosis outbreaks revealed several naturally occurring c74.22-negative strains with deficiency in glycosylation of the TA of the cell wall and resistance to serotype 4b-specific phage (4). Such surface antigen variants may arise in the course of the infection, representing an immune system evasion strategy on the part of the pathogen. However, one cannot exclude the possibility that they arose in the food vehicle or during subsequent passages of the bacteria in the laboratory.

Although c74.22-negative strains can be generated by transposon mutagenesis in the laboratory (11, 15), their emergence under laboratory conditions has not yet been described. Furthermore, it is not known whether such variants can arise in strains of ECII, which were not recognized prior to the 1998-1999 hot dog outbreak. In this study, we identified and characterized such variants of ECI and ECII strains, which arose in the course of laboratory investigations of these strains.

In the course of investigations of a genomic region ("region 18") which is located immediately upstream of *inlA*-*inlB* and which has diverged in ECII strains (5), we constructed a mutant of the ECII strain H7550 which harbored a deletion of region 18 and was designated ECIIΔ18R (3). The mutant was characterized by colony immunoblot assays with the MAb c74.22 described elsewhere (8). For these immunoblots bacteria were routinely grown in liquid, using as inoculum agar-grown cultures stored at 4°C. ECIIΔ18R (later designated ECIIΔ18RV) was found to be negative with MAb c74.22, whereas the parental strain H7550 reacted normally (Table 1; Fig. 1). However, when the −80°C stock culture of ECIIΔ18R was examined, it was found to be c74.22 positive, suggesting that the c74.22-negative phenotype was not due to the deletion. The c74.22-negative variant ECIIΔ18RV has remained negative with c74.22 in several repeated colony immunoblot tests (data not shown).

In a separate study, the ECI strain F2381, implicated in the California outbreak of 1985, was used to construct mutants of ECI-specific genes. In the process of characterizing several ECI-specific genes, we discovered that the deletion mutants, along with the parental strain F2381L (a streptomycin-resistant derivative of F2381), were negative with MAb c74.22. This finding was unexpected, since F2381L was earlier reported to be positive with c74.22 (11, 15). Repeated colony immunoblot assays with MAb c74.22 confirmed that F2381L (later designated F2381L-Phi-R) lacked reactivity with c74.22. However, the −80°C-preserved stock culture of F2381L maintained intact reactivity with this antibody (Table 1).

An earlier study revealed that the c74.22-negative epidemic-associated strains were all resistant to serotype 4b-specific phage (4). To determine whether the ECIIΔ18RV and F2381L-Phi-R variants may also have altered phage susceptibility profiles in comparison to their parental counterparts, infection and phage adsorption assays with the serotype 4b-specific phage A500 and the *Listeria* genus-specific phages A511 and Φ20422-1 (9, 12, 13) were carried out as described before (17). ECIIΔ18RV was resistant to one of these phages, Φ20422-1, whereas its parental counterpart ECIIΔ18R and the original wild-type strain H7550 were both susceptible (Table

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TABLE 1. Phage susceptibilities and c74.22 reactivities of strains investigated in this study

Strain	c74.22 reactivity[f]	Phage susceptibility[e]		
		Φ20422-1	A500	A511
F2381L[a]	+	S	S	S
F2381L-Phi-R	−	R	R	R
F2381L-Phi-R::pPL95	−	R	R	R
F2381L-Phi-R::pPLAB	−	R	R	R
F2381L-Phi-R::pPL2	−	R	R	R
H7550[b]	+	S	S	S
ECIIΔ18R[c]	+	S	S	S
ECIIΔ18RV[d]	−	R	S	S
ECIIΔ18RV::pPL95	−	R	S	S
ECIIΔ18RV::pPLAB	W	R	S	S
ECIIΔ18RV::pPL2	−	R	S	S

[a] Streptomycin-resistant derivative of F2381, ECI strain implicated in California outbreak (15).
[b] ECII strain from the 1998-1999 hot dog outbreak (5).
[c] Mutant of H7550 with deletion of region 18 (3), with intact reactivity with c74.22 and phage susceptibility.
[d] c74.22-negative variant derived from ECIIΔ18R.
[e] S, sensitive; R, resistant.
[f] −, negative; +, positive; W, weak.

TABLE 2. Adsorption deficiency of phage-resistant variants

Strain	Phage adsorption (PFU/ml)[a]		
	Φ20422-1	A511	A500
F2381L	1.75×10^4	1.92×10^3	5.42×10^3
F2381L-Phi-R	3.29×10^5	2.90×10^4	2.50×10^4
ECIIΔ18R	5.5×10^4		
ECIIΔ18RV	4.12×10^5		

[a] Adsorption of phages was measured by determining the number of PFU remaining in the supernatant of a mixture containing the indicated phage and strain, as described in the text. Results are averages from two independent experiments.

1). F2381L-Phi-R was resistant to infection not only with Φ20422-1 but also with the serotype 4b-specific phage A500 and the *Listeria* genus-specific phage A511. In contrast, the original F2381L strain (stock culture preserved at −80°C) was susceptible to all three phages (Table 1). Thus, the phage susceptibility profiles of the two variants were different from those of their parental counterparts but also differed markedly from each other.

Phage resistance may reflect changes in the presence or accessibility of phage receptors (18) or other mechanisms subsequent to infection. Adsorption of phage Φ20422-1 onto F2381L-Phi-R and ECIIΔ18RV was reduced 18.8-fold and 7.5-fold, respectively. Similar results were obtained with phage A511 and A500 adsorption onto F2381L-Phi-R (Table 2). These results suggest that loss of phage receptors accounted for the phage resistance of the variants. The receptors for phages A511 and A500 have been reported to be peptidoglycan and glycosylated TA, respectively (18). Thus, our findings that ECIIΔ18RV was susceptible to A511 but resistant to Φ20422-1 suggest that these two *Listeria* genus-specific phages use different receptors for infection of the bacteria. Phage receptors that may be absent in F2381L-Phi-R are currently unidentified.

With the exception of c74.22 reactivity and phage susceptibility, no other noticeable phenotypic differences could be detected between the variants and their c74.22-positive counterparts. Pulsed-field gel electrophoresis (PFGE) fingerprinting with AscI and ApaI, conducted as described elsewhere (6), revealed indistinguishable profiles in F2381L-Phi-R and F2381L (Fig. 2A). ECIIΔ18RV and ECIIΔ18R also had indistinguishable PFGE profiles with ApaI (Fig. 2B), as well as with AscI (data not shown).

Genetic complementation experiments with *gtcA* and *gltA-gltB*, previously shown to be required for reactivity of serotype 4b bacteria with c74.22 (11, 15), were pursued to determine whether the phenotypic alterations of the variants involved

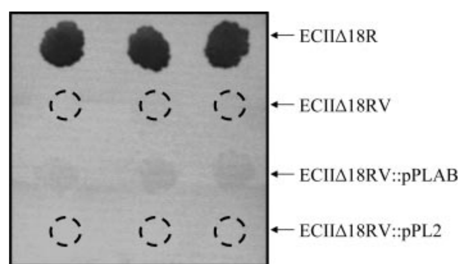

FIG. 1. *cis* complementation of surface antigen expression of ECIIΔ18RV with wild-type *gltA-gltB*. Overnight cultures were spotted on the nitrocellulose membrane in triplicate. From top to bottom are shown ECIIΔ18R (positive control; deletion mutant derived from strain H7550), ECIIΔ18RV (c74.22-negative variant of ECIIΔ18R), ECIIΔ18RV::pPLAB (ECIIΔ18RV harboring pPLAB), and ECIIΔ18RV::pPL2 (negative control; ECIIΔ18RV harboring cloning vector).

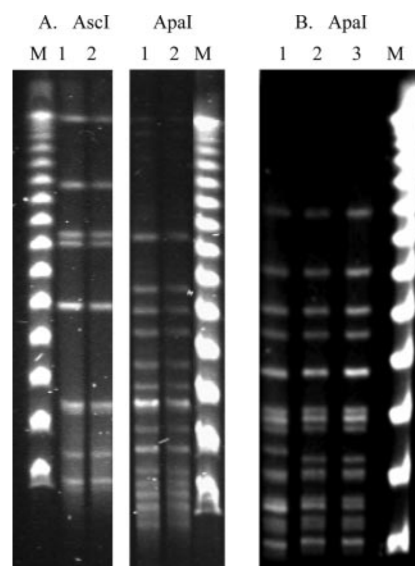

FIG. 2. PFGE profiles identical between c74.22-negative variants and their parental strains. (A) PFGE patterns of F2381L and F2381L-Phi-R generated by AscI (left) and ApaI (right). Lanes 1, F2381L (Str[r] derivative of F2381 [ECI]); lanes 2, F2381L-Phi-R (c74.22-negative laboratory variant of F2381L). (B) PFGE patterns of H7550, ECIIΔ18R, and ECIIΔ18RV generated by ApaI. Lane 1, H7550 (*Listeria monocytogenes* 4b wild type [ECII]); lane 2, ECIIΔ18R (deletion mutant derived from strain H7550); lane 3, ECIIΔ18RV (c74.22-negative laboratory variant of ECIIΔ18R). Lanes M, lambda concatemers (48.5 to 727.5 kb; New England Biolabs) used as molecular size markers.

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mutations in these genes. The DNA fragment harboring *gtcA* and its promoter region (300 nucleotides upstream of the start codon of *gtcA*) was amplified by PCR (primers VCpNP95F, 5′-ATAAGCGGCCGCTTCAAAGGGACAGGCAACATG, NotI site underlined, and VCpNP95R, 5′-ATAACCCGGGGTACTCAGGATGAATTCCAG, XmaI site underlined) with H7550 DNA as template and subcloned into the site-specific phage integration vector pPL2 (10) to generate pPL95. A DNA fragment containing *gltA-gltB* and the putative promoter region (219 nucleotides upstream of the start codon of *gltA*) was generated by PCR (primers VC2P3, 5′-AGTAGAGCTCGTAACGTCTCATATAGGGAG, SacI site underlined, and VC-1R5, 5′-CTCTGTCGACGTAGAACAATTGTAGTACCG, SalI site underlined) using H7550 DNA as template and was ligated to pPL2 to generate pPLAB. In order to introduce a single copy of *gtcA* or *gltA-gltB* into the genome of *Listeria monocytogenes*, pPL95 or pPLAB was transformed into *Escherichia coli* S17-1 (16), which was then conjugated with the c74.22-negative variants F2381L-Phi-R and ECIIΔ18RV as described previously (10). The resulting *gtcA*-complemented derivatives of F2381L-Phi-R and ECIIΔ18RV were designated F2381L-Phi-R::pPL95 and ECIIΔ18RV::pPL95, respectively. pPLAB harboring the *gltA-gltB* cassette was introduced into F2381L-Phi-R and ECIIΔ18RV, resulting in F2381L-Phi-R::pPLAB and ECIIΔ18RV::pPLAB, respectively. Partial restoration of reactivity with c74.22 was obtained in ECIIΔ18RV harboring the integrated pPLAB, whereas ECIIΔ18RV harboring the integrated pPL2 alone remained negative with this MAb (Fig. 1). Reactivity of F2381L-Phi-R with c74.22 was not recovered by either integrated pPL95 or pPLAB (Table 1).

The lack of restoration of reactivity of F2381L-Phi-R with the cloned *gtcA* (pPL95) was not likely to involve impaired expression of the cloned gene. In studies with the *gtcA* transposon mutant M44 (15), we found that the *gtcA* construct used here (pPL95) fully restored c74.22 reactivity and phage susceptibility to this mutant (Y. Cheng and S. Kathariou, unpublished data). Furthermore, the *gtcA* sequence of F2381 is identical to that in strain 4b1, from which M44 was derived, suggesting that strain-specific sequence differences were not responsible for the lack of complementation in F2381L-Phi-R. The *gltA-gltB* cassette sequences (including the upstream region) cloned in pPLAB were identical between the strain used as template to amplify the cassette (H7550) and F2381 (data not shown). Taken together, the current data suggest that F2381L-Phi-R harbors a mutation in a currently unidentified gene (other than *gtcA* or the *gltA-gltB* cassette) required for reactivity with c74.22.

Phage sensitivity of F2381L-Phi-R to A500, A511, or Φ20422-1 was not restored by either integrated pPL95 or pPLAB. ECIIΔ18RV also failed to recover phage susceptibility to Φ20422-1 with either integrated construct. Lack of restoration with the integrated pPLAB was surprising, since ECIIΔ18RV with integrated pPLAB had partial complementation in reactivity with c74.22 (Table 1; Fig. 1). It is possible that the number of receptor determinants in the pPLAB-complemented variant was insufficient or that their presentation at the cell surface was suboptimal. However, we cannot exclude the possibility that this strain harbors more than one mutation.

The conditions leading to emergence and establishment of serotype 4b variants such as described here remain unidentified. Under certain laboratory conditions the variants may have enhanced fitness in comparison to their parental strains. The bacteria remained c74.22 negative following multiple laboratory passages and, in the case of F2381L-Phi-R, following independent construction of mutants harboring deletions in several different genes, suggesting that the c74.22-negative phenotype of the variants is stable under laboratory conditions.

The identification of these variants in both ECI and ECII strains suggests that they do not represent isolated, exceptional events. It is conceivable that similar variants can arise and become inadvertently established in other serotype 4b strains. The current findings suggest that great care should be exercised in characterization of serotype 4b strains and genetic constructs thereof. Phenotypic attributes of the spontaneous variants (phage resistance, lack of reactivity with c74.22, and possibly impact on pathogenicity) could be erroneously attributed to the loss of the gene of interest in deletion mutants and other constructs. It would, therefore, be important for researchers to monitor the existence of 4b-specific surface antigens to avoid complication of their findings by spontaneous loss of the antigens. In our opinion, phage susceptibility assays with phages such as Φ20422-1 would be an ideal way for laboratories to monitor maintenance of the 4b-specific antigens in their strains when 4b-specific MAbs such as c74.22 are not available.

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