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Introduction of pAMβ1 into *Listeria monocytogenes* by Conjugation and Homology Between Native *L. monocytogenes* Plasmids

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The broad host range antibiotic resistance plasmid pAMβ1 was transferred from *Streptococcus faecalis* to 9 of 15 *Listeria monocytogenes* strains by conjugation. *L. monocytogenes* transconjugates could transfer the plasmid either among *L. monocytogenes* strains or back to *S. faecalis*. Transfer between the various strains occurred without any detectable plasmid DNA rearrangements. The pAMβ1 replicon was stable in *L. monocytogenes*—it was retained without antibiotic selection when the bacteria were grown in culture media or passed in mice—and the presence of pAMβ1 had no major effect on *L. monocytogenes* virulence. These data suggest that pAMβ1 or its derivatives might serve as useful *L. monocytogenes* cloning vehicles. The data presented also demonstrate that pAMβ1 is compatible with two different native *L. monocytogenes* plasmids and that *Listeria* species harbor native plasmids in addition to the 38.5-megadalton plasmid pRYC16 previously reported by Pérez-Díaz et al. (J. C. Pérez-Díaz, M. F. Vicente, and F. Banquero, Plasmid **8**:112–118, 1982). Of 29 *L. monocytogenes* strains screened, 7 contained plasmid DNA. Four strains had similar if not identical plasmids that were 34 megadaltons in size, whereas three other strains contained either a 53-, 44-, or 32-megadalton plasmid; none of these plasmids has the same restriction pattern as pRYC16. DNA homology experiments indicate that the various plasmids are related and suggest that there may be a common set of sequences present in all of the plasmids examined.

Listeria monocytogenes is a facultative intracellular parasite capable of causing disease in humans and animals. This organism has been extensively used in studies of the host immunological response to infection because it elicits a potent cell-mediated immune response (1, 2, 9, 12). However, very little is known about the basic mechanisms that allow virulent strains to survive and multiply inside macrophages while avirulent strains are killed. A number of possible *L. monocytogenes* virulence factors have been described, including hemolysin production (8, 13), nicotinamide adenine dinucleotidase activity (16), superoxide dismutase activity (20), and an endotoxin-like substance (21). Their role in *L. monocytogenes* intracellular survival and virulence, however, is unclear.

At present the study of *L. monocytogenes* virulence factors is hampered by a lack of defined genetic systems developed for this organism; neither transduction nor transformation has been described. Recently, however, Pérez-Díaz et al. (14) reported that the streptococcus plasmid pIP501 could be transferred from *Streptococcus* to *Listeria* species by conjugation. However, it has not been established whether pIP501 is stable in *L. monocytogenes* or whether this plasmid alters *L. monocytogenes* virulence.

In this report we describe experiments that suggest that the *S. faecalis* broad host range plasmid pAMβ1 (4) or derivatives of it might serve as useful cloning vehicles for *L. monocytogenes*. Our results also extend the findings of Pérez-Díaz et al. (14) to show that *L. monocytogenes* isolates contain plasmids in addition to the 38.5-megadalton (MDa) plasmid pRYC16, that these additional plasmids are related to each other, and that they may share a common set of DNA sequences.

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MATERIALS AND METHODS

Bacterial strains and media. *S. faecalis* JH2-2(pAMβ1) was obtained from D. Clewell (University of Michigan, Ann Arbor). *L. monocytogenes* strains were obtained as follows: 3A, 3B, 10403, and 61-1536 from stock cultures of the Department of Bacteriology and Public Health, Washington State University; strain 286-2 from the University of Oregon Health Sciences Center; strains B-53 and E-33 from the Hospital Clinical Microbiology Department, University of Washington; strain SH-12 from Sacred Heart Hospital, Spokane, Washington; and strains 78-Li89 and 81-Li63 from M. Cohen, Centers for Disease Control, Atlanta, Ga.

Bacterial strains were grown on brain heart infusion agar (BHI) (Difco Laboratories, Detroit, Mich.) or in BHI broth at 37°C. Antibiotics (purchased from Sigma Chemical Co., St. Louis, Mo.) used for selection of pAMβ1 and counterselection of donors were used at the following concentrations (μg/ml): erythromycin, 15; rifampin, 100; and streptomycin, 600.

Antibiotic-resistant mutants. *L. monocytogenes* cells (10 ml) were grown overnight, pelleted, suspended in 0.5 ml of BHI, and plated on BHI plates. A 20-μl portion of either streptomycin (150 μg/μl of distilled water) or rifampin (100 μg/μl of methanol) was pipetted onto the center of the plate. Colonies growing at the highest concentration of antibiotic were transferred to another BHI plate, and the appropriate antibiotic was again added. Single colonies from the highest antibiotic concentration were selected for use in mating experiments.

Virulence testing. Groups of five BALB/c mice each were injected intravenously (i.v.) with fivefold dilutions of log-phase *L. monocytogenes*. Mice were observed for 10 days, after which time no more deaths occurred. The 50% lethal dose was calculated by the method of Reed and Muench (15).

Mating procedure. For filter matings, equal volumes of donor and recipient bacteria from overnight cultures were mixed together, and 100 to 200 μl was transferred to a sterile Millipore GSTF filter on a BHI agar plate. After incubation overnight at 37°C, the organisms on the filter were suspended in 1 ml of BHI, and appropriate dilutions of the mating mixtures were spread on BHI antibiotic selection plates. Controls consisting of either the donor or the recipient alone were also treated in the above manner. Conjugation frequencies were expressed as the number of transconjugants per donor CFU. Colonies were counted after 48 h.

Preparation of *L. monocytogenes* DNA. A 10-ml portion of overnight bacterial growth in BHI was centrifuged at 11,700 × *g* for 10 min, washed in 5 ml of 0.1× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), suspended in 1 ml of 0.01 M sodium phosphate buffer in 20% sucrose (pH 7.0) with lysozyme (2.5 mg/ml), and incubated 45 min at 37°C. A 9-ml portion of lysis buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA, 500 μg of pronase B per ml [Calbiochem, La Jolla, Calif.], 1% sodium dodecyl sulfate) was then added. After an additional 30 min at 37°C, the samples were deproteinized by extraction with phenol and chloroform, and the nucleic acids were precipitated with ethanol. The samples were suspended in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA and stored at 4°C.

Plasmid DNAs. Plasmid DNA was prepared by a modification of the Birnboim and Doly technique (3). Overnight cultures (200 ml) were centrifuged at 13,300 × *g* for 10 min, and the cells were washed with 5 ml of 0.1× SSC, suspended in 9 ml of 0.01 M sodium phosphate in 20% sucrose (pH 7.0) with lysozyme (5 mg/ml), and incubated 45 min at 37°C. A 1-ml portion of Solution I (50 mM glucose, 100 mM EDTA, 250 mM Tris-hydrochloride [pH 8.0]) and 20 ml of Solution II (0.2 N NaOH–1% sodium dodecyl sulfate) were then added, and the mixture was held on ice for 5 min. A 15-ml portion of Solution III (3 M sodium acetate, pH 4.8) was added, followed by 30 min on ice with occasional mixing. The preparation was centrifuged at 11,700 × *g* for 15 min, and the plasmid DNA was precipitated from the supernatant with ethanol. The precipitate was then suspended in 4 ml of 50 mM Tris-hydrochloride–5 mM EDTA–50 mM NaCl and purified by banding in cesium chloride-ethidium bromide density gradients.

Restriction enzyme digests and agarose gel electrophoresis. *Eco*RI was isolated as described previously (5) and used in a reaction buffer consisting of 10 mM Tris-hydrochloride (pH 7.5)–150 mM NaCl–10 mM $MgCl_2$. Digested DNAs were analyzed by electrophoresis in 1% agarose gels in Tris-borate buffer (90 mM Tris-hydrochloride, 2.5 mM EDTA, 90 mM boric acid) at 110 V for 2.5 h. DNA bands were visualized by ethidium bromide staining followed by illumination with UV light. Lambda phage DNA digested with *Hin*dIII was used as molecular mass standard.

In vitro labeling of DNA. Plasmid DNA was labeled with [α-^{32}P]deoxyCTP (New England Nuclear Corp., Boston, Mass.) by the nick-translation reaction (11) as modified by Thomashow et al. (19).

Blot hybridization. Blot hybridizations were done by the method of Southern (17) as modified by Thomashow et al. (19). DNA was transferred from a single gel to two nitrocellulose sheets as previously described (10). Blots were washed in 0.3× SSC and 0.1% sodium dodecyl sulfate at 64°C. *L. monocytogenes* plasmids were assumed to be 38% G + C, the approximate composition of the genome (18). Therefore, the stringency of the wash was approximately Tm − 9°C (melting temperature − 9°C), which corresponds to about 6% base-pair mismatch (7).

RESULTS

Transfer of pAMβ1 from *S. faecalis* to *L. monocytogenes*. The broad host range plasmid pAMβ1, originally identified in *S. faecalis*, has been shown to transfer into at least nine streptococcal species, *Staphylococcus aureus*, *Lactobacillus casei*, and *Bacillus subtilis* (4). We therefore felt that this plasmid might be transferred to and replicate in *L. monocytogenes* and, if so, that it might be modified to serve as a cloning vector for *L. monocytogenes*. To determine whether the former was true, we carried out conjugation experiments in which *S. faecalis* JH2-2(pAMβ1) served as the donor strain and various *L. monocytogenes* isolates were used as recipients. The results indicated that transfer occurred with 9 of 15 *L. monocytogenes* strains tested (judged by the acquisition of erythromycin resistance). Frequency of transfer ranged from 10^{-4} to 10^{-8}. The six recipient strains which exhibited the highest frequencies are listed in Table 1. Plasmid transfer was examined in three strains (10403, SH-12, and 3B) and was found to be DNase resistant and nontransmissible with donor filtrates, indicating that conjugation was the probable mode of pAMβ1 transfer.

TABLE 1. Frequency of conjugative transfer of pAMβ1[a]

Recipient	Transfer frequency for:			
	S. faecalis JH2-2(pAMβ1)	*L. monocytogenes* SH-12(pAMβ1)	*L. monocytogenes* 10403(pAMβ1)	*L. monocytogenes* 3B(pAMβ1)
S. faecalis JH2-2	ND[b]	5.6×10^{-8}	6.3×10^{-7}	1.1×10^{-7}
L. monocytogenes 10403	4.4×10^{-4}	5.8×10^{-7}	4.1×10^{-6}	1.8×10^{-7}
L. monocytogenes SH-12	3.5×10^{-5}	5.4×10^{-8}	1.3×10^{-6}	2.2×10^{-7}
L. monocytogenes 3B	7.6×10^{-5}	4.2×10^{-8}	1.4×10^{-8}	2.7×10^{-7}
L. monocytogenes 78-Li89	1.1×10^{-7}	2.6×10^{-7}	1.3×10^{-6}	5.7×10^{-8}
L. monocytogenes 3A	7.2×10^{-7}	1.0×10^{-6}	1.1×10^{-7}	2.6×10^{-6}
L. monocytogenes B-53	1.1×10^{-7}	6.5×10^{-8}	5.4×10^{-8}	1.5×10^{-6}

[a] In the experiments in which *S. faecalis* JH2-2(pAMβ1) was the donor strain, the recipient *L. monocytogenes* strains were spontaneous streptomycin-resistant mutants (resistant to 600 μg/ml) of the indicated *L. monocytogenes* parental isolate. When the donors were *L. monocytogenes* strains, the *L. monocytogenes* recipients were spontaneous rifampin mutants (resistant to 100 μg/ml) of the indicated parental *L. monocytogenes* isolate, and the *S. faecalis* JH2-2 recipient was selected for by using rifampin (100 μg/ml). The spontaneous mutation frequencies of the recipient strains to erythromycin resistance were less than 10^{-10}. The spontaneous mutation frequency of *S. faecalis* JH2-2(pAMβ1) to streptomycin resistance and that of *L. monocytogenes* to rifampin resistance were less than 10^{-10}.

[b] ND, Not done. The frequency of transfer of pAMβ1 among *S. faecalis* strains was shown to be 10^{-2} by Hershfield (6).

Transfer of pAMβ1 from *L. monocytogenes* to *L. monocytogenes* and from *L. monocytogenes* to *S. faecalis*. *L. monocytogenes* cells which received pAMβ1 from *S. faecalis* at frequencies ranging from 4.4×10^{-4} to 1.1×10^{-7} (strains SH-12, 10403, and 3B) were used as donors to determine whether the plasmid could be transferred among *L. monocytogenes* strains and back to *S. faecalis*. The results from these experiments (Table 1) indicate that pAMβ1 transfer from *L. monocytogenes* to *L. monocytogenes* did occur at frequencies ranging from 4.1×10^{-6} to 1.4×10^{-8} and that *L. monocytogenes* to *S. faecalis* transfer ranged from 1.1×10^{-7} to 5.6×10^{-8}. The fact that pAMβ1 could be transferred out of *L. monocytogenes* strains that had initially received the plasmid from *S. faecalis* suggests that no major rearrangement of the plasmid occurred upon transfer and propagation in *L. monocytogenes* (also see below).

Plamid stability and effect on *L. monocytogenes* virulence. *L. monocytogenes* 10403 (serotype 1) is a virulent strain which is the prototype used in our laboratory to investigate *L. monocytogenes* pathogenicity and the activation of the host cellular immune response. This strain was tested for pAMβ1 plasmid stability and virulence. After some 55 generations of growth without antibiotic selection in BHI broth, 96% of the bacteria retained the erythromycin resistance phenotype as assayed by replica plating. This same strain was also tested for plasmid stability in BALB/c mice. After three animal passages, plate counts from spleen homogenates on both selective and nonselective media indicated that all of the recovered bacteria were still erythromycin resistant. The introduction of pAMβ1 into *L. monocytogenes* was not accompanied by a major alteration in the virulence of these organisms. The mouse 50% lethal dose of strain 10403 was 1.4×10^4, and after pAMβ1 acquisition it was 2.8×10^4.

***L. monocytogenes* plasmid DNA and compatibility with pAMβ1.** Pérez-Díaz et al. (14) reported that 7 of 32 *L. monocytogenes* isolates contained an identical 38.5-MDa plasmid which they named pRYC16. We wanted to determine whether any of our strains contained pRYC16 or other plasmids and, if so, whether pAMβ1 was compatible with the native plasmids. We examined 29 strains of *L. monocytogenes* and found that 7 contained plasmid DNA. *Eco*RI digests of these plasmids are shown in Fig. 1A. Four of the strains (3A, 286-2, 61-1536, and E-33) apparently contain the same plasmid, whereas the other three strains harbor different plasmids. None of these plasmids has the same *Eco*RI, *Bam*HI, or *Bgl*II restriction patterns described for pRYC16 (the *Bam*HI and *Bgl*II restriction patterns are not shown). Agarose gel electrophoresis of uncut plasmid DNA showed only one molecular species of plasmid in each of our strains (data not shown). The molecular mass of the various plasmids is calculated from the restriction fragments to be approximately: 34 MDa for p286-2, pE-33, p61-1536, and p3A; 53 MDa for pSH-12; 32 MDa for p81-Li63; and 44 MDa for p78-Li89.

Southern blot hybridizations were done to investigate the relatedness among the *L. monocytogenes* plasmids. p3A hybridized with all the *Eco*RI fragments of p286-2, pE-33, and p61-1536, further demonstrating that these plasmids are very closely related if not identical (Fig. 1B). The observation that p3A also hybridized with several *Eco*RI fragments of pSH-12, p81-Li63, and p78-Li89 (Fig. 1B) raised the possibility that the *L. monocytogenes* plasmids contained a core of related DNA sequences. Consistent with this notion is the observation that p78-Li89 hybridized with the largest *Eco*RI fragments of p286-2, pE-33, p3A, p61-1536, and p81-Li63 and two large *Eco*RI fragments of pSH-12 (Fig. 1C), all of which also hybridized with p3A.

Various *L. monocytogenes* transconjugants were examined for both pAMβ1 and their respective native plasmids to determine whether or not the *L. monocytogenes* plasmids were compatible with pAMβ1. This was accomplished by isolating total DNA from the transconjugants, digesting the samples with *Eco*RI, and preparing Southern blots of the digests. The blots were then hybridized with ^{32}P-labeled pAMβ1 or p3A. The results indicate that strains 286-2(pAMβ1), 3A(pAMβ1), and 78-Li89(pAMβ1) contained both pAMβ1 and the corresponding *L. monocytogenes* plasmid (Fig. 2). Thus pAMβ1 is compatible with p78-Li89 and the p3A family of plasmids. We do not yet know whether pAMβ1 is compatible with pSH-12 or p81-Li63. The observation that the pAMβ1 probe did not hybridize with DNA

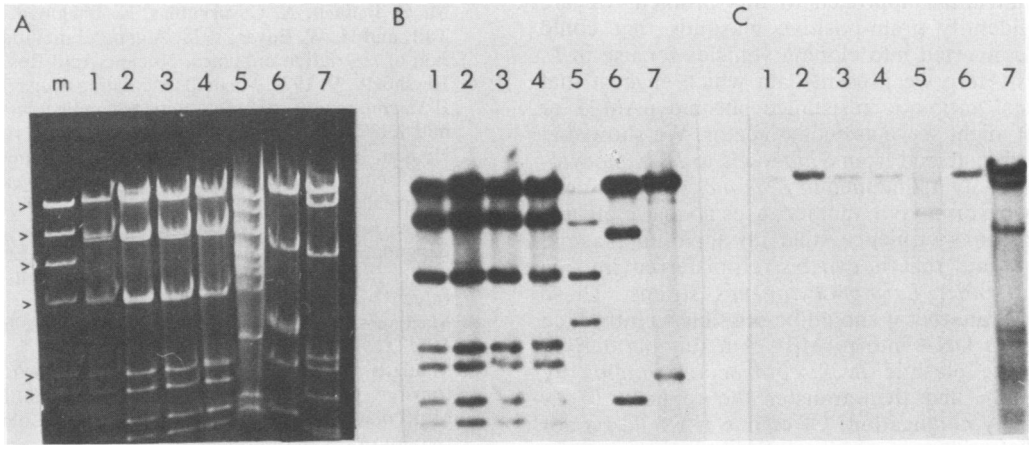

FIG. 1. Relatedness of native *L. monocytogenes* plasmids. Plasmid DNAs isolated from various *L. monocytogenes* strains were digested with *Eco*RI and fractionated by agarose gel electrophoresis. Lanes 1 through 7: p3A, p286-2, pE-33, p61-1536, pSH-12, p81-Li63, and p78-Li89, respectively. Lane m: *Hin*dIII-digested lambda phage DNA as molecular size standards (the sizes of the indicated fragments in kilobase pairs are 23.7, 9.4, 6.7, 4.2, 2.2, and 2.0). (A) Photograph of ethidium bromide-stained gel. (B) Autoradiogram of Southern blot prepared from a duplicate gel and hybridized with ^{32}P-labeled p3A. (C) Same as (B) except that the probe was ^{32}P-labeled p78-Li89.

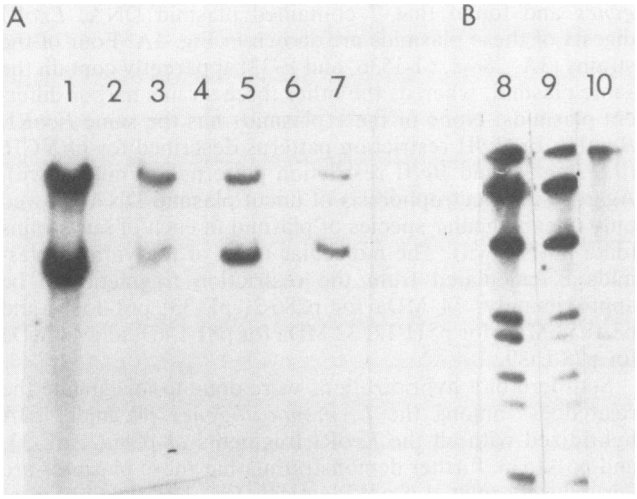

FIG. 2. Compatibility of pAMβ1 with native *L. monocytogenes* plasmids. Total DNAs were isolated from *L. monocytogenes* strains and digested with *Eco*RI. Southern blots were prepared and hybridized with either ^{32}P-labeled pAMβ1 (A) or p3A (B). Lanes 2 through 10: DNA from strains 286-2 (lane 2), 286-2(pAMβ1) (lanes 3 and 8), 3A (lane 4), 3A(pAMβ1) (lanes 5 and 9), 78-Li89 (lane 6), and 78-Li89(pAMβ1) (lanes 7 and 10). Lane 1: pAMβ1 isolated from *S. faecalis* JH2-2 and digested with *Eco*RI.

isolated from strains 286-2, 3A, or 78-Li89 indicates that pAMβ1 does not share closely related sequences with p286-2, p3A, or p78-Li89. We have also done hybridizations under low-stringency conditions (melting temperature − 39°C) and still have not detected homology between pAMβ1 and any of the native *L. monocytogenes* plasmids (data not shown). Finally, the fact that the *Eco*RI digest pattern of pAMβ1 appears to be unchanged in strains 286-2(pAMβ1), 3A(pAMβ1), and 78-Li89(pAMβ1) again suggests that it can be transferred to and propagated in *L. monocytogenes* without DNA deletions or rearrangements.

DISCUSSION

The study of *L. monocytogenes* virulence factors is severely limited by the unavailability of genetic systems for the organism. As an initial approach to this problem we are attempting to identify gram-positive plasmids that could potentially be converted into cloning vehicles for use in *L. monocytogenes*. Here we present data which suggest that the streptococcal antibiotic resistance plasmid pAMβ1 or derivatives of it might serve as such a vector. We show that pAMβ1 can be transferred from *S. faecalis* to *L. monocytogenes*, that it is stably maintained in *L. monocytogenes* cells propagated in vitro or in vivo, that it causes no major change in *L. monocytogenes* virulence, that no apparent plasmid deletions occur, and that it can be retransferred into *S. faecalis* or into other *L. monocytogenes* strains. These observations indicate that it should be possible to introduce *L. monocytogenes* DNA into pAMβ1 or a suitable derivative, establish the plasmid in *Streptococcus sanguis* by transformation (4), and then transfer the replicon to *L. monocytogenes* by conjugation. Of course it would be advantageous to introduce recombinant DNA directly into *L. monocytogenes*; however, this must await the development of a transformation procedure.

It has previously been shown that pIP501 can be introduced into *Listeria* species (14). This streptococcus plasmid also has a broad host range and codes for erythromycin resistance. It differs from pAMβ1 in that it has different *Hin*dIII and *Hin*cII restriction endonuclease patterns and it includes a gene coding for chloramphenicol resistance (6). Thus the relationship between these two plasmids is unclear.

In this report we also show that *L. monocytogenes* has at least four native plasmid species that differ from the pRYC16 plasmid previously described by Pérez-Díaz et al. (14). The plasmids which we have isolated are related, and it is possible that they share a common set of DNA sequences. The roles that these plasmids have in *L. monocytogenes* physiology or pathogenicity are unknown. However, their presence in strains isolated from geographically diverse areas (New York, Washington, Oregon, and Canada) over a 20-year period argues that they do have some function in *L. monocytogenes* survival in nature or perhaps in *L. monocytogenes* virulence. Further experiments are required to address this point.

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