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Molly C. Sutherland
Washington University School of Medicine in St. Louis

Joseph P. Vogel
Washington University School of Medicine in St. Louis

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Reclamation of Ampicillin Sensitivity for the Genetic Manipulation of *Legionella pneumophila*

Molly C. Sutherland and Joseph P. Vogel

Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA

Research on *Legionella pneumophila*, the causative agent of Legionnaires' disease, has been hampered due to the lack of selectable markers for genetic manipulation. We report the construction of a mutant strain of *L. pneumophila* lacking *loxA*, a chromosomally encoded β-lactamase, that has enhanced sensitivity to ampicillin. Also described are a method for converting *Legionella* strains to ampicillin sensitivity and conditions for utilizing *bla* as a selectable marker.

Legionellae are ubiquitous freshwater bacteria that are able to replicate within protozoa in the environment and can also grow inside alveolar macrophages (6, 8, 12). Human infection can lead to a form of pneumonia called Legionnaires' disease, which was first identified during an outbreak at an American Legion convention held in Philadelphia, PA, in 1976 (17). Legionnaires' disease was traditionally treated with the macrolide erythromycin, and more recently with fluoroquinolones, due to their membrane-permeant nature and the absence of drug resistance (6, 12–14, 19). In contrast, β-lactams are not used to treat Legionnaires' disease, congruent with the observation that *Legionella pneumophila* strains commonly display a low level of resistance to β-lactams *in vitro* and are highly resistant *in vivo* (3, 7, 10, 15, 18, 22).

Consistent with this result, it has recently been shown that many *Legionella* species encode at least one β-lactamase. For example, the *L. pneumophila* strain Philadelphia I was shown to encode a single class D β-lactamase, *loxA* (*Legionella* oxacillinase) (2), which is homologous to the well-characterized β-lactamase OXA-29 in *Legionella gormanii* (5, 9, 11, 16) (see Fig. S1 in the supplemental material). *loxA* was proposed to be solely responsible for the β-lactamase activity present in the *L. pneumophila* strain Philadelphia I, since lysates from this strain and from an *Escherichia coli* strain expressing *loxA* contained a similar β-lactamase profile (2). However, since a strain lacking *loxA* had not been constructed, it was not possible to definitively state that *loxA* was accountable for all of the β-lactamase activity observed in this strain.

Endogenous resistance to ampicillin of most *L. pneumophila* strains has precluded the use of β-lactamase as a selectable marker for basic research, thus compounding the lack of selectable markers currently available for *L. pneumophila* basic research. Thus, with the goals of confirming that LoxA is responsible for the β-lactam resistance of the *L. pneumophila* Philadelphia I strain and potentially developing an ampicillin-sensitive version of this strain that could be used for genetic manipulation, we engineered a Δ*loxA* mutant in Lp02, a commonly used derivative of *L. pneumophila* Philadelphia I (see the supplemental Materials and Methods and Fig. S1 in the supplemental material) (4).

In order to determine if the loss of *loxA* increased the sensitivity of a *Legionella* strain to ampicillin, the growth of strain Lp02 (JV4918) was compared with that of a Δ*loxA* mutant (JV4921) on plates containing increasing concentrations of ampicillin (0, 0.125, 0.25, 0.5, 1, 2, 4, and 10 μg/ml ampicillin). Lp02 is resistant to small amounts of ampicillin, and limited growth can be observed on plates containing 10 μg/ml ampicillin (Fig. 1B, top left quadrant). In contrast, a strain lacking *loxA* is quite sensitive to ampicillin and cannot grow on plates having low levels of ampicillin (Fig. 1B, bottom left quadrant of plate containing 0.125 μg/ml ampicillin). Similar results were observed when comparing the growths of two additional commonly used *L. pneumophila* strains, JR32 and AA100 (1, 20), with their corresponding *loxA* deletions on plates containing ampicillin (data not shown). Therefore, *loxA* appears to be solely responsible for the ampicillin resistance of *L. pneumophila* Philadelphia I as previously proposed (2).

Having generated a strain with increased sensitivity to ampicillin, we examined whether β-lactamase (*bla*) could be used as a selectable marker in *L. pneumophila* by comparing the growth of the Δ*loxA* mutant (JV4921) with that of the Δ*loxA* mutant expressing *bla* from a plasmid (JV4920). In contrast with the ampicillin sensitivity of the Δ*loxA* mutant without *bla* expression, expression of *bla* allowed the Δ*loxA* mutant to form normal-sized colonies on plates containing 0.5 to 1.0 μg/ml ampicillin (Fig. 1B, bottom right quadrant). It is worth noting that selection for a *bla*-expressing plasmid was not feasible in the strain Lp02 as it conferred only a slight increase in growth on ampicillin plates (Fig. 1B, compare top left and top right quadrants). Based on these results, we can conclude that the strain Lp02 is naturally resistant to ampicillin, that deletion of *loxA* results in a strain that has increased sensitivity to ampicillin, and that expression of *bla* in the *loxA* deletion strain is able to restore ampicillin resistance, thus allowing *bla* expression to be used as a selectable marker in this *Legionella* background.

Since *L. pneumophila* grown in broth exhibits more-uniform growth and infectivity than bacteria grown on solid medium, broth-grown cultures are often preferentially used for experiments. Therefore, the replication of JV4921 (Δ*loxA*) and JV4920 (Δ*loxA* + *bla*) grown overnight in liquid medium containing increasing amounts of ampicillin was examined. Growth was mea-

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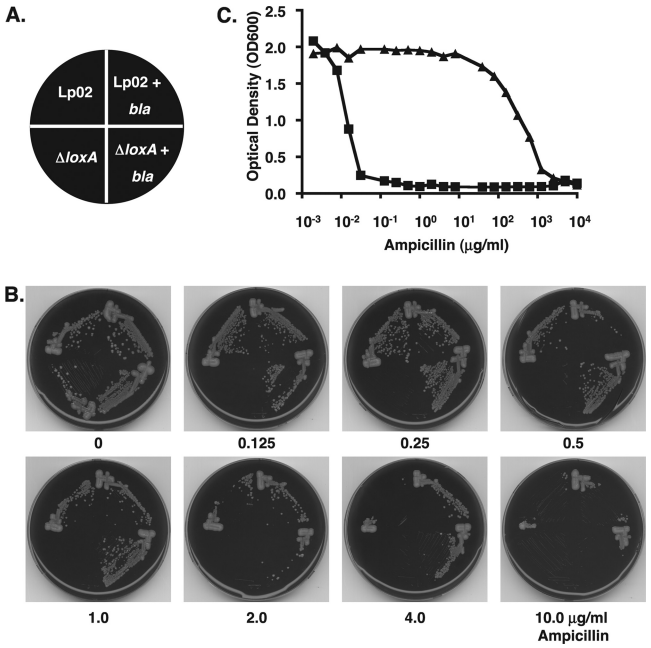

FIG 1 Ampicillin can be used as a selectable marker. (A and B) The strains JV4918 (Lp02), JV4919 (Lp02 + *bla*), JV4920 (Δ*loxA* + *bla*), and JV4921 (Δ*loxA*) were streaked for isolated colonies on solid bacteriologic medium containing 0, 0.125, 0.25, 0.5, 1, 2, 4, or 10 μg/ml ampicillin. (C) JV4921 (squares) and JV4920 (triangles) were inoculated in liquid medium containing 2-fold dilutions of ampicillin ranging from 10 mg/ml to 0.002 μg/ml. The cultures were grown overnight, and the optical density at 600 nm (OD_{600}) was measured the next day. The data are representative of three independent experiments.

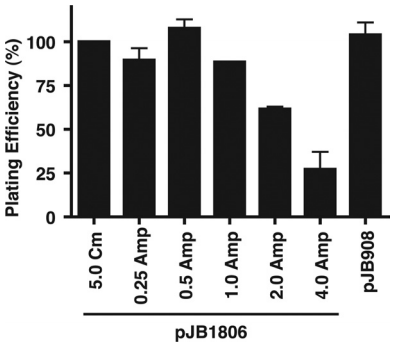

FIG 2 Effects of ampicillin concentration on plating efficiency. A Δ*loxA* strain (JV3690) was transformed with 1 μg of pJB1806 (ampicillin-resistant [Amp^r], Cm^r plasmid). Transformants were plated on solid medium containing 0.25, 0.5, 1, 2, or 4 μg/ml ampicillin or chloramphenicol. Transformation of a second plasmid, pJB908, was selected on plates lacking thymidine and used as an independent control for transformation frequency. Plating efficiency on ampicillin was determined by comparing growth on solid medium containing ampicillin to growth on chloramphenicol. Transformations were done in triplicate, and the error bars represent the standard deviations from the mean.

sured the next day by optical density readings at 600 nm (OD_{600}) and plotted as a function of ampicillin concentration (Fig. 1C). Replication of JV4921 was completely inhibited by 0.125 μg/ml ampicillin, whereas full growth inhibition of JV4920 required 2,500 μg/ml ampicillin (Fig. 1C). Moreover, JV4920 was able to grow in the presence of up to 8 μg/ml ampicillin at a rate comparable to that in the absence of the drug (Fig. 1C). Thus, 0.5 μg/ml ampicillin in plates and 5 μg/ml ampicillin in broth are recommended as reasonable concentrations to select for a *bla* plasmid in the Δ*loxA* mutant.

Although 0.5 μg/ml ampicillin in solid medium was sufficient to inhibit the growth of the Δ*loxA* mutant, it was worth determining if larger amounts of the drug could be used when selecting for a *bla*-expressing plasmid during a transformation. To investigate this, the plating efficiency of a Δ*loxA* strain transformed with the plasmid pJB1806, which confers both ampicillin and chloramphenicol resistance (Cm^r), was examined. The number of transformants obtained on ampicillin plates was normalized to the number observed on plates containing chloramphenicol (set to 100% in Fig. 2). Consistent with our previous results after streaking the bacteria for isolated colonies (Fig. 1B), 0.5 μg/ml ampicillin did not affect the plating efficiency of the Δ*loxA* strain expressing *bla*. However, increasing the ampicillin concentration 4- or 8-fold (to 2 or 4 μg/ml) resulted in a significant decrease in the plating efficiency of the strain (Fig. 2). Therefore, we recommend using 0.5 μg/ml ampicillin as the optimal concentration of the drug to select for *bla* when transforming the *L. pneumophila* Lp02 Δ*loxA* strain.

A key feature of *Legionella* virulence is the ability to replicate within host cells, making it important to confirm that deleting *loxA* did not indirectly affect intracellular survival and growth of this pathogen. Replication was assayed within two host cells, *Acanthamoeba castellanii* and A/J mouse-derived bone marrow macrophages. Growths were compared for strain Lp02 (JV4933), a Δ*loxA* strain (JV4934), and a type IV secretion system-deficient mutant (JV4936) (see Fig. S2 in the supplemental material). Fold growth was monitored at defined time points by plating on charcoal yeast extract (CYE) plates supplemented with thymidine and chloramphenicol (see Fig. S2A and C in the supplemental material) or ampicillin (see Fig. S2B and D in the supplemental material). Importantly, the Δ*loxA* strain (JV4934) and the Lp02 strain (JV4933) grew similarly, indicating that a strain lacking *loxA* is not attenuated for intracellular growth. Moreover, the number of CFU obtained on ampicillin plates was the same as that on chloramphenicol plates, demonstrating that ampicillin can function as a new selectable marker for monitoring intracellular replication of *L. pneumophila*.

Having demonstrated that *bla* can be used as a selectable marker for *L. pneumophila* research, it was important to develop a simple and efficient method for the construction of ampicillin-sensitive strains. Our initial Δ*loxA* strains used in this study were constructed through a standard loop-in/loop-out procedure (see the supplemental Materials and Methods in the supplemental material). Although this protocol is effective, it can be labor-intensive and time-consuming. In contrast, construction of deletions by natural transformation requires less work and is more rapid (21). To optimize the natural transformation reaction, a Δ*loxA*::Cm^r suicide plasmid (pJB3998) that contains 500 bp of DNA on each side of the *loxA* locus was constructed and tested. Natural transformations were performed as previously described (21) and involved spotting DNA onto a newly made patch of *L. pneumophila*, growing the strain for 2 days at 30°C, and streaking for isolation. By selecting for chloramphenicol resistance, Δ*loxA*::Cm^r transformants were obtained by natural transformation of Lp02 using 0.01 to 10 μg of pJB3998 (Fig. 3). The transformants were authentic, as spontaneous chloramphenicol

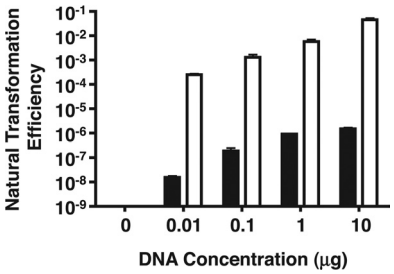

FIG 3 Δ*loxA* natural transformants can be obtained at high frequencies using a suicide plasmid containing 4 kb of DNA surrounding the *loxA* locus. pJB3998 (0.5 kb of flanking DNA) and pJB5283 (4 kb of flanking DNA) were used to naturally transform Lp02 with increasing DNA concentrations of 0.01 to 10 μg. Natural-transformation frequencies were determined by dividing the number of natural transformants (Cm^r) by the number of viable cells. The numbers of transformants obtained with various amounts of DNA are shown for pJB3998 (filled bars) and for pJB5283 (open bars). Transformations were done in triplicate, and the error bars represent the standard deviations from the mean.

col-resistant colonies were never detected when water was used in the transformation reaction (Fig. 3).

Although transformants were obtained, frequencies higher than 1/100,000 were not achieved, even when using 10 μg of DNA. This low frequency precludes the construction of an unmarked deletion by this method because identification of the deletion would be exceedingly laborious. As a result, a chloramphenicol marked-natural transformation vector (pJB5283) that contained 4 kb of DNA flanking the *loxA* gene was made, as a previous report indicated that lengthening the size of the homologous DNA resulted in higher transformation efficiencies (21). Significantly more transformants were obtained using pJB5283 than using pJB3998, resulting in a transformation frequency of approximately 4% when 10 μg of pJB5283 was used (Fig. 3). This frequency should be sufficiently high to allow direct detection of an integration of an unmarked vector in a natural transformation reaction.

To confirm that an unmarked deletion can be made in one step using this method, we repeated the natural transformations using pJB3951, which is an unmarked Δ*loxA* plasmid containing 4 kb of homologous flanking DNA. Transformants were identified by comparing growths on CYET plates with and without 0.5 μg/ml ampicillin. Using pJB3951, we have been able to achieve transformation efficiencies approaching 2.5%. This high level of integration will allow for the efficient identification of Δ*loxA* transformants by a significantly faster method than the classical loop-in/loop-out strategy, although the latter strategy can still be used in strains that are not naturally competent. In addition, our protocol is not dependent on leaving a second drug marker on the chromosome, a result discordant with the development of an additional selectable marker.

In conclusion, unmarked deletions of *loxA* that are sensitive to ampicillin can now be easily generated via a single step in naturally competent forms of *L. pneumophila*. This will be beneficial to researchers studying *Legionella* pathogenesis, as only a few antibiotics (e.g., kanamycin and chloramphenicol) are commonly used in the lab setting. This problem has been exacerbated by endogenous resistance to some antibiotics and/or inactivation of some antibiotics, including tetracycline, by the charcoal-yeast extract medium used to culture this pathogen (7). Therefore, reclamation of ampicillin as an additional selectable marker will be useful in many of the *L. pneumophila* strains that have been constructed in laboratories around the world.

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