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Reclamation of Ampicillin Sensitivity for the Genetic Manipulation of Legionella pneumophila

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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 inside 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 ΔloxA 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 ΔloxA 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 ΔloxA mutant (JV4921) with that of the ΔloxA mutant expressing bla from a plasmid (JV4920). In contrast with the ampicillin sensitivity of the ΔloxA mutant without bla expression, expression of bla allowed the ΔloxA 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 ΔloxA 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 (ΔloxA) and JV4920 (ΔloxA + bla) grown overnight in liquid medium containing increasing amounts of ampicillin was examined. Growth was mea-
phenicol resistance (Cmr), was examined. The number of trans-
plasmid pJB1806, which confers both ampicillin and chloram-
this, the plating efficiency of a
ing if larger amounts of the drug could be used when selecting for
hibit the growth of the
rable 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 recom-
med as reasonable concentrations to select for a bla plasmid in the ΔloxA mutant.
Although 0.5 µg/ml ampicillin in solid medium was sufficient to inhibit the growth of the ΔloxA mutant, it was worth determin-
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 ΔloxA strain transformed with the plasmid pJB1806, which confers both ampicillin and chloramphenicol resistance (Cmr'), 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 streak-
ing the bacteria for isolated colonies (Fig. 1B), 0.5 µg/ml ampicillin did not affect the plating efficiency of the ΔloxA 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 ΔloxA strain.

FIG 2 Effects of ampicillin concentration on plating efficiency. A ΔloxA strain (JV3690) was transformed with 1 µg of pJB1806 (ampicillin-resistant [Ampr], Cmr' plasmid). Transformants were plated on solid medium containing 0.25, 0.5, 1, 2, or 4 µg/ml ampicillin or chloramphenicol. Transformation of a sec-
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 trip-
llicate, and the error bars represent the standard deviations from the mean.

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. Growth was monitored at defined time points by plating on charcoal yeast extract (CYE) plates supplemented with thymidine and chloramphenicol (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 mate-
or ampicillin (see Fig. S2B and D in the supplemental mate-
Importantly, the ΔloxA 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 ΔloxA strains used in this study were constructed through a standard loop-in/loop-out procedure (see the supplemental Materials and Methods in the supplemental ma-
Although this protocol is effective, it can be labor-intensive and time-consuming. In contrast, construction of deletions by
moral clones on solid bacteriologic medium containing 0, 0.125, 0.25, 0.5, 1, 2, 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 µg/ml to 0.002 µg/ml. The cultures were grown overnight, and the optical density at 600 nm (OD600) was measured the next day. The data are representative of three independent ex-
periments.

FIG 1 Ampicillin can be used as a selectable marker. (A and B) The strains JV4918 (Lp02), JV4919 (Lp02 + bla), JV4920 (ΔloxA + bla), and JV4921 (ΔloxA) were streaked for isolated colonies on solid bacteriologic medium containing 0, 0.125, 0.25, 0.5, 1, 2, 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 µg/ml to 0.002 µg/ml. The cultures were grown overnight, and the optical density at 600 nm (OD600) was measured the next day. The data are representative of three independent ex-
periments.
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 ΔloxA 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 ΔloxA 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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