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Generation of Targeted Nonpolar Gene Insertions and Operon Fusions in *Pasteurella haemolytica* and Creation of a Strain That Produces and Secretes Inactive Leukotoxin

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An efficient method for targeted gene inactivation and generation of chromosomal gene fusions in *Pasteurella haemolytica* has been devised and used to create an *lktC*::*cat* operon fusion by allelic exchange at the leukotoxin gene cluster (*lktCABD*). A copy of the *lktC* gene was insertionally inactivated by using a nonpolar, promoterless *cat* cassette and then delivered into *P. haemolytica* on a shuttle vector. Plasmid incompatibility was used to detect clones where double recombination events had occurred at the chromosomal locus. The insertion in *lktC* did not affect expression of the downstream genes, and the mutant strain secreted an antigenic proleukotoxin that was neither leukotoxic nor hemolytic. Expression of the *lktC* gene in *trans* restored the wild-type phenotype, confirming that LktC is required for activation of the proleukotoxin to the mature leukotoxin. Construction of the *lktC*::*cat* operon fusion allowed us to quantitate leukotoxin promoter activity in *P. haemolytica* and to demonstrate that transcription was maximal during early logarithmic growth phase but was reduced following entry into late logarithmic phase. This allelic exchange system should be useful for future genetic studies in *P. haemolytica* and could potentially be applied to other members of *Haemophilus-Actinobacillus-Pasteurella* family, where genetic manipulation is limited.

Pasteurella haemolytica is a major causative agent of bovine shipping fever pneumonia (14) and produces several factors important for induction of the disease. Leukotoxin is considered to be the primary virulence factor of *P. haemolytica* (31, 35), but it is clear that there are other potential factors involved in its pathogenesis, including lipopolysaccharide, polysaccharide capsule, fimbriae, glycoprotease, neuraminidase, serotype-specific antigen, and outer membrane proteins (6). The leukotoxin is a member of the genetically related family of bacterial cytolysins termed repeats in toxin (43) and is most similar to the *Actinobacillus pleuropneumoniae* and *Escherichia coli* hemolysins (15, 39). The leukotoxin operon encodes the leukotoxin (LktA) and proteins required for its activation (LktC) and secretion (LktB and LktD) (4, 19, 26). Reciprocal *trans* complementation in *E. coli* heteroplasmid systems has been used to correlate leukotoxin genes and functions (13, 20), but similar experiments have not been performed in *P. haemolytica.*

Analysis of leukotoxin and other virulence genes in *P. haemolytica* is limited by a lack of efficient tools for generating defined chromosomal mutations. Development of electroporation and conjugation techniques for *P. haemolytica* (8) made it possible to perform allelic exchange by the commonly used methodology of Gutterson and Koshland (17). This strategy was based on strong positive selection for mutations and involved use of a suicide plasmid that carried the gene of interest insertionally inactivated with a selective marker. With this technique, only three different chromosomal loci of *P. haemolytica* have been inactivated (24, 28, 29, 40), all at very low frequency. The presence of stringent restriction systems (2, 21) and a low frequency of homologous recombination with respect to illegitimate recombination (28) have made the positive selection method cumbersome and inefficient in *P. haemolytica*, especially in the absence of a simple phenotypic screen. These problems necessitated the development of more effective methods for gene replacement in *P. haemolytica*.

Despite advances in allelic exchange technology, working cloning vectors were not available for *P. haemolytica* until recently. The difficulties in creating a genetic system have been due, largely, to a lack of expressed selective markers and broad-host-range plasmids that can be used in this organism. A small ampicillin-resistant (Ap^r) plasmid of *P. haemolytica* was examined as a potential shuttle vector (1, 45), but the plasmid was difficult to manipulate. We have created a set of *P. haemolytica-E. coli* shuttle cloning vectors derived from another native plasmid, pYFC1 (3). These vectors utilize a variety of antibiotic resistance markers and have been used successfully for gene cloning and expression in *P. haemolytica* (12).

Development of the shuttle vectors allowed us to devise a positive-negative selection approach for allelic exchange in *P. haemolytica*, using two incompatible, nonsuicide plasmids. In this method, the mutagenic plasmid, carrying the gene of interest insertionally inactivated with a selective marker, is propagated in *P. haemolytica* to allow the recombination to occur. A second incompatible plasmid is then introduced to displace the mutagenic plasmid. The approach uses three different antibiotic resistance genes: a vector marker, a mutagenic marker, and a marker for the second incompatible plasmid. The mutagenic marker that we used was a promoterless chloramphenicol acetyltransferase gene (*cat*) carried on a nonpolar cassette that confers chloramphenicol resistance (Cm^r) only when transcribed from an upstream promoter (27). The cassette was chosen for this system for the following reasons: (i) the *cat* gene can be expressed in *P. haemolytica* when transcribed by *P. haemolytica* promoters (12), (ii) insertion of the cassette within an operon does not affect expression of downstream genes (27), (iii) chloramphenicol acetyltransferase (CAT) is a convenient reporter enzyme for measuring gene expression in operon and protein fusions (34), and (iv) most *P. haemolytica*

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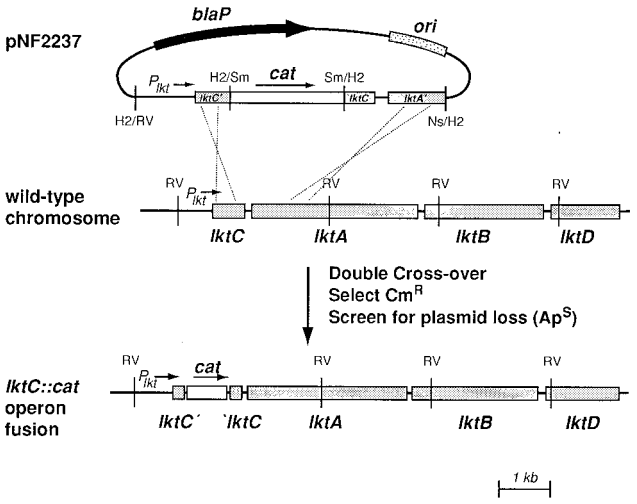

FIG. 1. Experimental strategy used to insertionally inactivate the chromosomal *lktC* gene with the *cat* cassette. pNF2237, carrying the inactivated *lktC* gene, was introduced into *P. haemolytica*, and then the incompatible plasmid, pYFC1, was transformed into the strain with selection for Sm^r (not shown). Double recombinants, where pNF2237 was lost but *cat* was retained, were identified as Cm^r Ap^s colonies. H2, *Hin*cII; Sm, *Sma*I; RV, *Eco*RV; Ns, *Nsi*I.

strains are Cm^s and do not exhibit detectable spontaneous resistance (8, 9). Thus, the *cat* gene should be very useful in studies of gene expression and function in *P. haemolytica*.

In this report, we used the positive-negative selection approach to perform allelic exchange at the *P. haemolytica* leukotoxin locus (*lktCABD*). Creation of an *lktC* mutant of *P. haemolytica* and subsequent *trans* complementation have allowed us to demonstrate that LktC is required for conversion of the proleukotoxin to the mature active toxin. Insertion of the *cat* gene at the leukotoxin locus has created an operon fusion that can be used to quantitate leukotoxin transcription in *P. haemolytica*.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q}*ZΔM*15 Tn*10*]; Stratagene) was used for plasmid propagation and cloning. *E. coli* was grown at 37°C in liquid or solid Luria-Bertani medium (33). *P. haemolytica* SH1217 was used in all other experiments (12). *P. haemolytica* was grown at 37°C in liquid or solid brain heart infusion (BHI) broth (Difco) or on 5% sheep blood agar plates. Antibiotics were used at the following concentrations: for *E. coli*, ampicillin at 50 μg/ml, chloramphenicol at 30 μg/ml, and streptomycin at 20 μg/ml; for *P. haemolytica*, ampicillin at 50 μg/ml, chloramphenicol at 5 to 10 μg/ml, and streptomycin at 100 to 500 μg/ml.

DNA manipulation. Standard recombinant DNA techniques were used (33). *E. coli* cells were transformed by electroporation (11). *P. haemolytica* cells were prepared and electroporated as previously described (8). As a shuttle vector, we used an Ap^r *P. haemolytica-E. coli* plasmid, pNF2176 (12), derived from a native streptomycin-resistant (Sm^r) plasmid, pYFC1 (3). pNF2176 is Ap^r Sm^s because the Sm^r gene was replaced with the Ap^r gene, *blaP*. pYFC1 and pNF2176 are incompatible because they both carry the same *ori*; and in the absence of selection, homoplasmid strains are easily segregated.

To create the mutagenic plasmid (Fig. 1), a plasmid, pNF2232, was first constructed by cloning the 2-kb *Eco*RV-*Nsi*I fragment of pSH224 (19), carrying *lktC* and the 5′ end of *lktA*, onto *Hin*cII-linearized pNF2176 (12). The promoterless nonpolar *cat* cassette was excised from pSL1 (27) by *Sma*I digestion (0.7 kb) and inserted into the *Hin*cII site within the *lktC* reading frame on pNF2232 to create pNF2237. Thus, on pNF2237, the *lktC* gene is insertionally inactivated and the leukotoxin promoter is fused to *cat*. Note that the *cat* gene contains the complete CAT polypeptide coding sequence but lacks a promoter and transcriptional terminator. Further, the *cat* open reading frame is preceded by translational stop codons in all three reading frames and is immediately followed by a consensus ribosome-binding site, GGAGG, and an ATG start codon at its 3′ end (27).

Selection of *lktC* strains and plasmid curing in *P. haemolytica*. Strain SH1217, carrying pNF2237, was electroporated with the incompatible plasmid pYFC1. Transformants were selected on plates containing streptomycin, and the presence of both autonomous plasmids was confirmed by replica plating onto ampicillin and chloramphenicol plates and by preparing and analyzing plasmids minipreps (data not shown). Transformants were pooled and propagated overnight in 5 ml of BHI containing 500 μg of streptomycin per ml to permit plasmid segregation. To select clones where *cat* was rescued by allelic exchange at the *lktCABD* locus, 100 μl of the overnight culture was spread on BHI plates containing 5 μg of chloramphenicol and 100 μg of streptomycin per ml. Then Sm^r Cm^r Ap^s double recombinants were detected by replica plating onto ampicillin plates.

P. haemolytica lktC mutants were cured of pYFC1 by propagating them overnight in BHI containing 20 μg of novobiocin (Sigma) per ml, then plating the culture for single-colony isolation. Sm^s colonies were detected by replica plating. Following this treatment, 75% of colonies had lost pYFC1.

Southern analysis of genomic DNA. *P. haemolytica* genomic DNA was isolated by using the DNAzol reagent (Life Technologies). One microgram of genomic DNA was digested overnight with 20 U of *Eco*RV, and fragments were separated by electrophoresis in a 0.8% agarose gel. Southern blotting was performed as previously described (19). The blot was hybridized with one of the following ^{32}P-labeled (Random Primed DNA labeling kit; Boehringer Mannheim) DNA fragments: the 2.0-kb *Eco*RV-*Nsi*I fragment of pSH224, the 0.7-kb *Sma*I fragment of pSL1, or *Hin*cII-linearized pNF2176.

Immunoblotting. *P. haemolytica* strains were grown at 37°C with rotatory shaking to an optical density at 600 nm (OD_{600}) of 1.0 in BHI broth. Preparation and immunoblotting of culture supernatants and cell lysates were performed as described elsewhere (19), with cell lysates being concentrated 10-fold with respect to supernatants. The blotted proteins were probed with monoclonal mouse antibodies (MM601, MM602, MM603, and MM605) (16) (kindly provided by S. Srikumaran) or with polyclonal bovine convalescent serum (19). Immune complexes were detected with biotin-conjugated goat anti-murine or goat anti-bovine antibodies (Kirkegaard and Perry Laboratories), followed by horseradish peroxidase-conjugated streptavidin and 4-chloro-1-naphthol (Sigma).

BL-3 cytotoxicity assays. *P. haemolytica* leukotoxin was prepared from culture supernatants as follows. Strains were grown at 37°C with rotatory shaking to mid-logarithmic phase (OD_{600} of 1.0) in BHI broth, and then cells were collected by centrifugation and resuspended in RPMI 1640 medium (Sigma), containing 3.5% bovine serum albumin, to an OD_{600} of 0.25. Cultures were incubated at 37°C with shaking to an OD_{600} of 1.0. Cells were pelleted by centrifugation at 12,000 × *g* for 30 min, and then the supernatant fluids were filtered through 0.2-μm-pore-size cellulose acetate filters and stored at −80°C. Leukotoxic activity was measured by leakage of lactate dehydrogenase (LDH) from bovine lymphosarcoma cells (BL-3; CRL 8037; American Type Culture Collection) as described elsewhere (5). BL-3 cells were resuspended in RPMI 1640 medium (5×10^5 cells/ml) and exposed for 2 h at 37°C to *P. haemolytica* culture supernatants from different strains, in a total volume of 250 μl. After incubation, unlysed cells were collected by centrifugation (2 min, 5,700 × *g*), and the supernatants were assayed for LDH spectrophotometrically at 340 nm and 25°C, using a commercial LDH substrate reagent (LD-L50; Sigma). One toxic unit is the amount of leukotoxin that caused 50% leakage of the total LDH activity from 5×10^5 BL-3 cells in 250 μl of RPMI 1640 medium after 2 h of incubation at 37°C. Triton X-100 (0.1%) was substituted for culture supernatants in the incubation mixture as a control for maximal leakage of LDH. Spontaneous leakage was determined from BL-3 cells incubated with RPMI 1640 medium alone.

CAT assays. The *lktC*::*cat* fusion strain (SH1562) or strain SH1217, carrying pNF2237, was grown at 37°C in BHI with rotatory shaking. At different time points on the growth curve, 1-ml culture samples were collected by centrifugation at 12,000 × *g* for 2 min, then cooled to 4°C, washed in 1 ml of 0.1 M Tris (pH 7.8), and finally resuspended in 1 ml of 1 mM dithiothreitol–0.1 M Tris (pH 7.8). Cells were sonicated for two 10-s bursts at 50 W and then centrifuged at 12,000 × *g* for 30 min at 4°C, to remove cell debris. Cell extracts were normalized with respect to OD_{600} and stored at −20°C. The CAT assay (30) was carried out in 3.5-ml glass miniscintillation vials. Two microliters of each cell extract was added to a 250-μl reaction mixture containing 100 mM Tris-HCl (pH 7.8), 1.0 mM chloramphenicol, and 0.1 mM [^{3}H]acetyl coenzyme A (200 mCi/mmol, 0.5 mCi/ml; DuPont NEN Research Products). The reaction mixture was gently overlaid with 3 ml of a water-immiscible scintillation fluor (Econofluor; Packard) and then incubated at 25°C. At selected time intervals, the individual vials were counted for 1 min.

RESULTS

Creation of *lktC* strains. Our strategy for allelic exchange in *P. haemolytica* was based on the assumption that we should be able to increase the probability of recombination by using a nonsuicide multicopy plasmid that could be maintained long enough to permit double-crossover events. Plasmid incompatibility was used to promote loss of the original mutagenic vector and to facilitate detection of mutants. To specifically

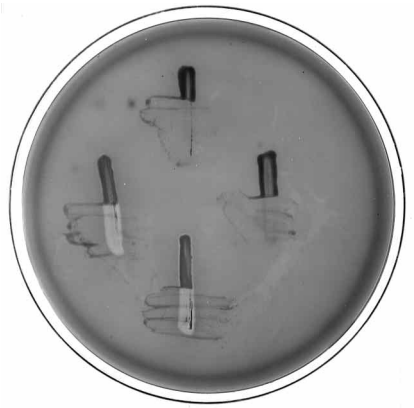

FIG. 2. Blood agar plate demonstrating the hemolytic phenotypes of *P. haemolytica* SH1562 (*lktC*) and the *trans*-complemented strain SH1562. Strains are, clockwise from the top, SH1562, SH1562 carrying pNF2176, SH1562 carrying pNF2232, and SH1217 (wild type). Strains were streaked on a blood agar plate. Following overnight growth at 37°C, a portion of the streak was removed with a cotton-tipped applicator to reveal the zones of hemolysis.

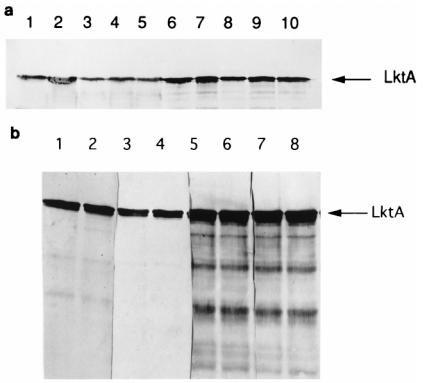

FIG. 3. Immunoblot analysis of leukotoxin expression in *P. haemolytica* with polyclonal bovine convalescent serum and with monoclonal murine antibodies. (a) The blotted proteins from supernatants (lanes 1 to 5) and whole-cell lysates (lanes 6 to 10) of *P. haemolytica* were developed with bovine serum. Lanes: 1 and 6, SH1562 (*lktC*); 2 and 7, SH1562 carrying pNF2232; 3 and 8, SH1562 carrying pNF2176; 4 and 9, SH1217 (wild type); 5 and 10, SH1217 carrying pNF2176. (b) The blotted proteins from supernatants of the wild-type (lanes 1, 3, 5, and 7) and mutant (lanes 2, 4, 6, and 8) strains were developed with antibodies MM605 (lanes 1 and 2), MM603 (lanes 3 and 4), MM601 (lanes 5 and 6), and MM602 (lanes 7 and 8). LktA indicates the position of leukotoxin.

inactivate the *lktC* gene within the *P. haemolytica* leukotoxin operon, we constructed plasmid pNF2237, carrying the *lktC* gene insertionally inactivated with the nonpolar *cat* cassette (Fig. 1). Plasmid pNF2237 (Ap^r Cm^r) was electroporated into *P. haemolytica* SH1217. Electroporation efficiency into this strain was extremely low (10 CFU/μg), suggesting that the cloned sequences contain sites for the *P. haemolytica* restriction system(s). Nevertheless, this allowed us to establish and maintain the plasmid in the strain. An isolate was propagated overnight in BHI to allow recombination to occur; then pYFC1 (Sm^r) was introduced into the strain. Transformants were pooled and again propagated overnight to allow segregation of markers. Sm^r Cm^r colonies were then characterized by replica plating onto plates containing ampicillin. Of 500 colonies screened, four Ap^s double recombinants were identified. To create an isogenic $LktC^-$ strain, pYFC1 was eliminated by curing with novobiocin. The strains were passaged for three days in BHI without chloramphenicol to verify that the *cat* gene was stably maintained.

The configuration of the insert in each strain was examined by Southern hybridization (data not shown). In each, the 3.1-kb *Eco*RV fragment carrying *lktC* was replaced by the inactivated copy corresponding to a fragment of 3.8 kb. This corresponds the size of the fragment plus the *cat* cassette. When the *cat* cassette was used as a probe, the same fragments hybridized with the probe in the *lktC* mutants, and no hybridization signals were detected for the wild-type strain. Vector DNA alone did not hybridize to DNA in any of the mutants. Thus, in each of the Sm^r Cm^r Ap^s isolates analyzed, double recombination had occurred at the leukotoxin locus. None contained replicon fusions between pNF2237 and pYFC1, and none resulted from single recombination events. One of the *lktC* mutants, called SH1562, was chosen for analysis in the following experiments.

***trans* complementation of hemolysis by *lktC*.** In the related *E. coli* hemolysin system, the HlyC protein functions as an acyltransferase that posttranslationally modifies and activates prohemolysin (25). The LktC protein in *P. haemolytica* is assumed to have a similar mode of action, but this has not yet been examined. Since leukotoxin is responsible for the hemolytic activity of *P. haemolytica* (29), inactivation of *lktC* should result in a nonhemolytic phenotype. This was observed (Fig. 2), further verifying that the Cm^r Ap^s isolates resulted from recombination at the leukotoxin locus. To provide direct evidence that LktC is required for leukotoxin hemolytic activity in *P. haemolytica* and to demonstrate the nonpolar character of the mutation in the *lktC* strain, we used the *lktC* plasmid, pNF2232, to complement the mutation (Fig. 2). Plasmid pNF2232, carrying a functional *lktC* gene, restored hemolytic activity to the *lktC* mutant, while pNF2176 did not. These results indicate that mature leukotoxin is required for the hemolysis observed in wild-type *P. haemolytica* strains.

Leukotoxin production by the *lktC* strain. Since the hemolytic phenotype was restored in the *lktC* strain by *trans* complementation, we inferred that proleukotoxin (pro-LktA) was produced by strain SH1562. To verify that the mutant strain produced and secreted pro-LktA, we performed immunoblotting with convalescent polyclonal bovine antibodies and with murine monoclonal antibodies from hybridomas MM601 (neutralizing), MM602 (neutralizing), MM603 (nonneutralizing), and MM605 (nonneutralizing) (16). The 102-kDa protein corresponding to leukotoxin was detected with the bovine convalescent serum in both supernatants and cell lysates from all *P. haemolytica* strains examined, including the wild-type strain, the mutant strain carrying the pNF2176 vector, the mutant carrying the complementing *lktC* plasmid, pNF2232, and the mutant alone (Fig. 3a). It appears that the *lktC* mutation does not significantly affect expression or secretion of the proleukotoxin because all of the strains produced and secreted the proteins at approximately the same level. These results also demonstrate that inactive pro-LktA can be efficiently secreted from the cells. The relative electrophoretic mobility of LktA and pro-LktA was similar in this gel system, and more protein was observed in the supernatants than in whole-cell samples, consistent with previous reports (4). Since the inactivation of *lktC* leads to the production of inactive toxin, it was of interest to determine if neutralizing monoclonal antibodies directed against the toxin would react with the proleukotoxin. Immunoblot analysis of supernatants from wild-type and mutant strains by using murine monoclonal antibodies (Fig. 3b) confirmed the results obtained with convalescent serum and demonstrated that pro-LktA is still recognized by leukotoxin-neutralizing antibodies MM601 and MM602. Thus, the neutralizing epitope is not restricted to the acyl group(s) on the

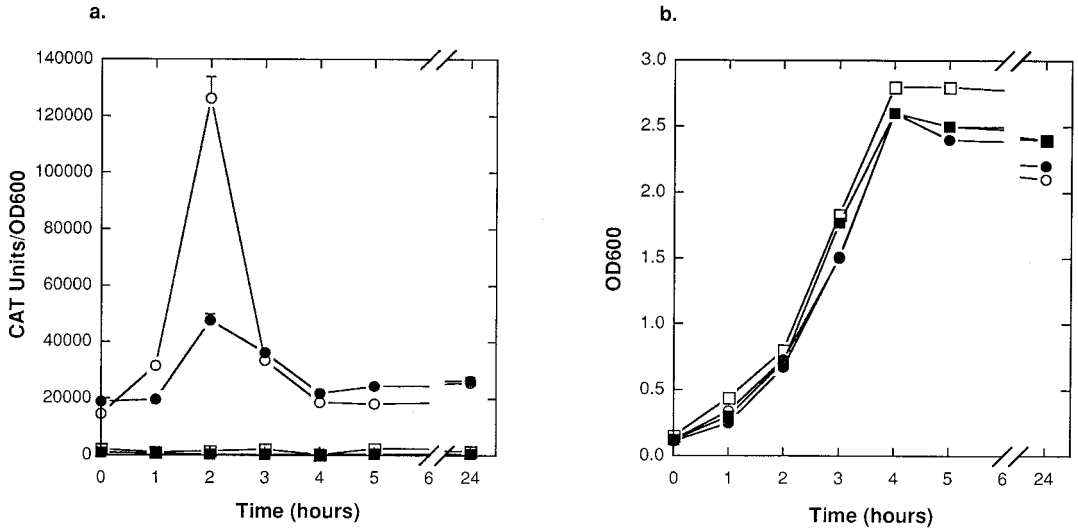

FIG. 4. *cat* gene expression from the leukotoxin promoter. (a) CAT activity was measured at various times across the growth curve in strains SH1562 (filled circles), SH1217 (open squares), SH1217 carrying pNF2176 (filled squares), and SH1217 carrying pNF2237 (open circles). CAT activity is reported as cpm per OD_{600} of the culture at the indicated time points. The results presented are averages for duplicate samples of a representative experiment. (b) Growth curves for the strains listed above.

mature toxin. The large number of lower bands observed in assays using MM603 and MM605 is consistent with that reported by others (16), and these bands are likely to be proteolytic products of the toxin.

Leukotoxic activity of the *lktC* strain. The *P. haemolytica* leukotoxin causes lysis of ruminant macrophages and other leukocytes (36). Subcytotoxic levels of leukotoxin may also impair pulmonary defenses and induce inflammatory responses (37). Since LktC is required to activate pro-LktA, we predicted that pro-LktA, secreted by strain SH1562, would not be cytotoxic. Leukotoxic activity was measured by using cultured bovine lymphosarcoma (BL-3) cells (5, 42) exposed to culture supernatants from mutant, wild-type, and *trans*-complemented strains. Supernatants from logarithmic cultures of the *P. haemolytica* wild-type strain contained 180 U of leukotoxic activity per ml, and a supernatant from the *trans*-complemented strain contained 60 U per ml. No leukotoxic activity was observed in supernatants from the mutant strain SH1562 (−10 U/ml), while Triton X-100 treatment yielded 300 U per ml. These results show definitively that expression of *lktC* is required for production of active leukotoxin by *P. haemolytica*, and they confirm previous findings for *E. coli* (13, 20). The failure to achieve full complementation in the mutant was unexpected since the vector is estimated to be present at 10 to 20 copies per cell and the complemented strain produces wild-type levels of LktA.

Growth phase-dependent expression of the leukotoxin promoter. The *cat* gene is an important reporter to study transcriptional regulation because it produces an enzyme that can be easily assayed with specificity and great sensitivity (34). We adapted the fluor diffusion assay (30) to measure CAT activity from the *lktC*::*cat* operon fusions on plasmid pNF2237 and on the chromosome (SH1562) (Fig. 4). *cat* expression from the leukotoxin promoter was growth phase dependent whether carried on the chromosome or on a plasmid. For each, a peak of activity occurred during the early logarithmic phase of growth, and activity rapidly declined to a steady-state level as the cells entered mid-logarithmic phase. A threefold-higher level of activity was observed in the multicopy plasmid state than in the single-copy state, but in stationary phase, both strains produced nearly equivalent levels of the enzyme. As mentioned above, based on copy number considerations, we had anticipated that the plasmid-encoded levels would be at least 10-fold higher than chromosomal levels.

DISCUSSION

A common strategy for allelic exchange is based on strong positive selection and uses mutagenic suicide plasmids that cannot replicate in the targeted recipient. Nevertheless, the technique is inefficient in *P. haemolytica* because of stringent restriction-modification systems, low frequency of transformation, and potentially rare homologous recombination. Without phenotypic selection, this method is especially cumbersome and involves screening thousands of colonies by colony hybridization and Southern hybridization to recover rare double recombinants (28, 29). Since *P. haemolytica* lacks the sophisticated and flexible genetic systems available for other organisms, we felt that some of the barriers to allelic exchange could be overcome by using nonsuicide plasmids. We returned our focus to the first site-directed mutagenesis experiments (32), where positive-negative selection and incompatible plasmids were used. Here, we applied the same strategy to specifically inactivate the *lktC* gene of *P. haemolytica*. A plasmid carrying the gene, insertionally inactivated with *cat*, was coestablished with an incompatible replicon. Plasmid segregation was used to detect clones where *cat* was rescued by homologous recombination at the leukotoxin locus.

Insertional inactivation of the *lktC* gene abrogated cytotoxicity. The mutant strain was neither leukotoxic nor hemolytic but produced and secreted proLktA that was still antigenic. Expression of the *lktC* gene in *trans* restored the wild-type phenotype, providing direct evidence that LktC is required for activation of *P. haemolytica* proleukotoxin and that leukotoxin is responsible for the hemolytic and leukotoxic effects of the organism. This was entirely consistent with previous results reported for *E. coli* hemolysin and *P. haemolytica* leukotoxin in *E. coli* (13, 20). To our knowledge, the experiment described here is also the first report of genetic complementation in *P. haemolytica*. Expression and secretion of the active leukotoxin

by a complemented mutant strain also indicated that the *cat* cassette had indeed created a nonpolar insertion.

The leukotoxin, which is produced by most *P. haemolytica* strains (3), is considered to be the primary virulence factor of *P. haemolytica* (31). It is also an important antigen in the protection against shipping fever (7). An LktC$^-$ LktA$^-$ strain, constructed earlier by marker exchange at the leukotoxin locus of *P. haemolytica*, was not cytotoxic, because it did not produce or secrete LktA (29). Our *lktC* mutant differs since it still produces the inactive leukotoxin plus other wild-type antigens but lacks cytotoxicity. The proleukotoxin reacts efficiently with leukotoxin-neutralizing antibodies and therefore should elicit neutralizing immune responses in cattle. As a result, the LktC$^-$ strain, producing genetically toxoided leukotoxin, may be useful for vaccine development. Future studies will be aimed at testing the virulence of the strain.

Generation of the chromosomal fusion to *cat* allowed us to assess leukotoxin promoter activity in *P. haemolytica*. Since the *P. haemolytica* leukotoxin genes are poorly expressed in *E. coli*, it was suggested that *Pasteurella*-specific transcriptional factors are required for effective expression (22). Several putative regulators of *P. haemolytica* leukotoxin expression have been identified by using an *E. coli trans*-complementation system based on *lktC*::*lacZ* gene fusions (18, 23), but until now, we have been unable to study their functions and activities in *P. haemolytica*. Here we used the *lktC*::*cat* gene fusion to monitor leukotoxin expression in *P. haemolytica* by measuring the level of acetylchloramphenicol produced in cell extracts. We observed that leukotoxin expression reached a maximum in early logarithmic phase and declined later as the cells entered late logarithmic and stationary phases. The early- to-late-logarithmic-phase expression ratio for the chromosomal fusion was about 2.5-fold, while the plasmid-borne fusion ratio was 6.5-fold. Increased leukotoxin expression in early logarithmic phase has been observed in Northern blots (38), but our results also revealed a decline in expression as the cells leave early logarithmic phase. Similar kinetics, with delayed appearance of the peak, were observed for secretion of *P. haemolytica* leukotoxin (42) and *E. coli* hemolysin (41) into cell supernatants. This is the first example of a chromosomal reporter gene fusion used to monitor gene expression in *P. haemolytica*. The relevance of our observations can be interpreted more fully when additional transcriptional data become available for this organism.

In summary, we have developed a new method for allelic exchange in *P. haemolytica* that has the following advantages: (i) it is very efficient because nonsuicide plasmids can be maintained in the target host long enough to ensure double recombination events, (ii) it requires only minimal knowledge of the molecular biology of target host and a simple genetic system, (iii) specific inactivation the gene of interest with the nonpolar selection cassette does not affect expression of downstream genes, and (iv) it creates a chromosomal fusion to *cat* that has been used as a reporter to quantitate promoter activity. This method should be useful for genetic studies and vaccine development in *P. haemolytica*. We hope that the system may function in other members of the *Haemophilus-Actinobacillus-Pasteurella* family of bacteria since plasmids similar to pYFC1 has been identified in *Actinobacillus pleuropneumoniae* (44), *Pasteurella multocida* (46), and *Haemophilus ducreyi* (10).

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