Note

Construction of a high-efficiency shuttle vector for *Histophilus somni*

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A B S T R A C T

The genetic manipulation of *Histophilus somni* is limited due to its high-fidelity restriction–modification system. The broad host-range shuttle plasmid pLS88 is capable of transforming some strains of *H. somni*, but is an inefficient vector. We have constructed an improved version of pLS88, pNS3K, that transforms *H. somni* strain 2336 100-fold more efficiently than its predecessor. The transformation efficiency was further increased when pNS3K was isolated from *H. somni* and retransformed into the same strain. As proof of principle, the lipooligosaccharide biosynthesis gene *lob-2A* was cloned into pNS3K and expressed in *H. somni* strain 129Pt, which lacks this gene. Thus, pNS3K is a useful shuttle vector for *H. somni* and a potential vector for genetic manipulation of this bacterium.

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Histophilus somni (*Haemophilus somnus*) (Angen et al., 2003), is a gram-negative, coccobacillus and member of the *Pasteurellaceae*. This host-specific, opportunistic pathogen of cattle is one of the organisms responsible for bovine respiratory disease complex (BRDC), or shipping fever (Corbeil et al., 1986). In addition to causing pneumonia, *H. somni* is responsible for thrombotic meningoencephalitis, myocarditis, arthritis, abortion, septicemia, and other systemic infections (Dierks et al., 1973; Inzana, 1999). The study of microbial pathogens depends on the capability of investigators to clone, express, and mutate putative virulence genes. Mutagenesis of *H. somni* using allelic exchange is difficult because of the apparently tight-restriction–modification system of this organism; only two genes have been mutated to date (Wu et al., 2000; Sanders et al., 2003). The only known shuttle vector that is capable of transforming *H. somni* is the broad host range vector pLS88, which originated from *Haemophilus ducreyi* (Dixon et al., 1994). However, this vector is large in size (4.8 kb) due to its excessive number of antimicrobial selection markers, has a limited number of available cloning sites, and is inefficient in transforming disease isolates of *H. somni*. As there is a need to improve genetic manipulation of *H. somni*, we have constructed a new improved vector to circumvent the drawbacks of pLS88 and to express proteins in *H. somni*.

H. somni strain 2336 (a pathogenic, pneumonia isolate) was used to determine the transformation and expression efficiency of the new shuttle vector. *H. somni* was propagated from −80 °C skim milk stocks onto Columbia agar containing 5% sheep blood (CBA). For electroporation, the bacteria were grown to a density of 120 Klett units (10^9 colony forming units (CFU)/ml) in brain heart infusion (BHI) broth supplemented with 0.01% thiamine monophosphate (TMP) and 10% Levinthal base at 37 °C with shaking at 200 rpm (Wu et al., 2000). The bacteria were chilled on ice for 30 min and harvested by centrifugation at 5000 ×g for 5 min. The cells were washed twice with 272 mM sucrose, and sedimented after each wash by centrifugation at 5000 ×g for 10 min. The bacteria were suspended in 272 mM sucrose to 1% of the original volume, and aliquots of 39 µl were used for electroporation after the addition of 300–500 ng of plasmid DNA. Electroporation was performed using a BTX ECM 600 electroporator (BTX, Inc., San Diego, Calif.) at 50 µF, 1.6 kV, and 200 ohms with a cuvette gap of 1 mm. Following electroporation the cells were diluted to 1 ml with BHI broth, chilled on ice for 10 min, incubated at 37 °C for 3 h, and spread onto antibiotic plates for selection of transformants. For maintenance of plasmids pLS88 and pNS3K in *H. somni*, 80 µg/ml of streptomycin or kanamycin were used in the growth media, respectively. For calculation of transformation efficiency, the number of *H. somni* colonies transformed with pNS3K and pLS88 was determined on selective media by viable plate counts. The transformation efficiency was further evaluated following plasmid isolation from *H. somni* and retransformation into the same strain.

DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis were performed according to standard techniques (Sambrook et al., 1989). Polymerase chain reactions (PCR) were performed using Platinum PCR SuperMix High Fidelity (Invitrogen,

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Carlsabad, CA) in a Gradient Mastercycler® (Eppendorf, Westbury, NY) according to the manufacturer's instructions. Each 50 μl of PCR reaction mix contained 45 μl of Super Mix, 200 nM of primers, and 100 ng of template DNA. Reactions were performed for 35 cycles with denaturation at 94 °C for 30 s, annealing was at different temperatures for different primers (Table 1) for 30 s, extension at 68 °C for 1 min, and final extension for 10 min at 72 °C. Oligonucleotides were purchased from Sigma-Genosys (Sigma). Restriction and modification enzymes were purchased from Promega. The QIAprep Spin Miniprep Kit from QIAGEN was used for all plasmid extraction and the QIAGEN PCR cleanup kit was used for all restriction enzyme removal and DNA gel extraction.

The kanamycin resistant gene promoter (KanPm) was amplified from the pUC4K cloning vector (Pharmacia) with primers KanPm-F and KanPm-R (Table 1). SalI and BamHI restriction sites were designed into the forward and reverse primers for directional cloning. Six histidine and one glycine residues were introduced following the translational start codon in the reverse primer KanPm-R to facilitate epitope tagging at the amino terminus (Seleem et al., 2004). To avoid translational errors or inhibition arising from rare codon bias (Makrides, 1996), the preferred codon usage of *H. somni* strain 129Pt, *H. ducreyi* strain 3500HP, and *Haemophilus influenzae* strain Rd (http://www.kazusa.or.jp/codon/cgi-bin/showcodon. cgi?species=731) for histidine and glycine were used. The KanPm was cloned into the promoterless vector pNS to fuse KanPm with downstream MCS (Seleem et al., 2004). The KanPm and the downstream MCS were later excised from the pNS vector using SalI and EcoRI restriction enzymes.

The kanamycin resistance gene (Kan^R) with its own promoter was amplified from the cloning vector pUC4K (Amersham Pharmacia) using Kan-F and Kan-R primers. EcoRI and SpeI sites were designed into the forward and reverse primers, respectively for directional cloning (Table 1). The ScaI and XbaI sites were designed into the Kan-F primer for use as additional, unique cloning sites within the MCS, followed by a strong transcriptional stop signal (UAAU) (Tate and Mannering, 1996; Collier et al., 2002). The origin of replication (ori) was amplified with its own promoter from the broad host range shuttle vector pLS88 (Willson et al., 1989; Dixon et al., 1994) using primers Ori-F and Ori-R. SalI and XbaI restriction sites were designed in the forward Ori-F and reverse Ori-R primers, respectively, for directional cloning. The XbaI site in the reverse Ori-R primer was deleted after cloning with the SpeI compatible restriction site of Kan^R. After restriction digestion and gel purification, a three-way ligation reaction was carried out by temperature cycle ligation (TCL) (Lund et al., 1996) in a Mastercycler Gradient thermocycler programmed to cycle between 30 s at 10 °C and 30 s at 30 °C for 12 h. The ligation of the KanPm and MCS, the Kan^R, and the ori gene formed plasmid pNS3K. The ligation mixture was transformed into *Escherichia coli* Mach 1 chemically competent cells (Invitrogen) and transformants were cultured onto

kanamycin (50 μg/ml) LB plates for selection. Colonies were picked after 10–12 h for screening and plasmid extraction. The T2 transcription terminator derived from *rrnB* in the rRNA operon of *E. coli* (Brosius et al., 1981) was amplified from the expression vector pTrcHis2A (Invitrogen, Carlsbad, CA) using primers rrnBT-F and rrnBT-R (Table 1). The ScaI and XbaI sites were designed in the rrnBT-F primer and SpeI in the rrnBT-R primer. The transcription terminator was cloned downstream of the MCS using ScaI and XbaI restriction sites (Fig. 1).

The lipooligosaccharide (LOS) biosynthesis gene *lob-2A*, with its own promoter, was amplified from pLSlob2A using primers *lob-2A*-F and *lob-2A*-R. The BamHI and XbaI sites were designed in the *lob-2A* forward primer and XbaI in the *lob-2A* reverse primer. pLSlob2A is a pLS88 vector containing intact *lob-2A* in a SmaI site, and was used in an earlier study to complement the lob2A glycosyl transferase in *H. somni* strain 129Pt (Wu et al., 2000). The amplified *lob-2A* was cloned into the BamHI and XbaI site of pNS3K to form pNS3K/lob2A, which was introduced into nonphase-variable *H. somni* strain 129Pt by electroporation, as described above to obtain strain 129Pt(pNS3K/lob2A). Transformants were selected on media containing 80 μg/ml kanamycin, and transformants were screened by PCR with the primers used to amplify *lob-2A*.

LOS was extracted from wild type and each recombinant *H. somni* strain following growth in broth using a hot phenol-water micro-extraction method (Inzana, 1983). LOS was separated and its electrophoretic profile determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through a 15% polyacrylamide separating gel containing urea and a bilayer stacking gel as previously described (Inzana and Apicella, 1999). LOS profiles were visualized by silver staining (Tsai and Frasch, 1982).

H. somni strain 2336 was selected for detailed study with pNS3K because the genome of this strain has been sequenced (http://microgen.ouhsc.edu/h_somnus/h_somnus_home.htm), and being a pathogenic strain, is more difficult to transform than non-pathogenic strains. The transformation efficiency of pNS3K was 100-fold (2 logs) greater than that of pLS88 (10^4 transformants/μg of pNS3K compared to 10^2 transformants/μg of pLS88). This increase in transformation efficiency of pNS3K was likely due to reduced size (Briggs and Tatum, 2005) and absence of redundant sequences in pNS3K compared to pLS88. When pNS3K was isolated from *H. somni* and retransformed into the same strain, the transformation efficiency increased and continued to increase with subsequent isolation and retransformation into the same strain.

The restriction–modification system of *Mannheimia haemolytica* has been reported to interfere with genetic exchange in this bacterium (Briggs et al., 1994). *H. somni* also has an apparently tight restriction–modification system, which has not been well-characterized, and commercially available methylases protect only one or more sites against cleavage by specific restriction enzymes (Sanders et al., 1997; Wu et al., 2000; Briggs and Tatum, 2005). Sanders et al. (1997) showed that passage of pLS88 in a rec-1 strain of *H. influenzae* before transformation into *H. somni* enhanced its transformation efficiency, further supporting our premise that endogenous methylation of the plasmid during each retransformation cycle results in protection of the DNA from enzyme degradation. However, passage of plasmids in strain 129Pt did not improve transformation efficiency in strain 2336, which is due to strain to strain differences in *H. somni* restriction–modification (RM) systems (Challacombe et al., 2007). *H. somni* strain 2336 contains a functional Type I and type II RM system as compared to strain 129Pt which has non-functional Type I and Type II RM systems (Siddaramappa, 2007).

To determine if genes cloned in pNS3K can be expressed in *H. somni*, the LOS biosynthesis gene lob2A (encoding for an *N*-acetylglucosamine transferase) was cloned into strain 129Pt, which lacks this gene and *N*-acetylglucosamine off heptose I in its LOS outer core (*N*-acetylglucosamine is present off heptose II in strain 129Pt (St. Michael et al., 2004). Previous expression of lob2A in strain 129Pt, followed by gel electrophoresis and NMR of LOS, and nano Electrospray-Mass Spectrometry

Table 1
PCR primers used in this study

Name	Primer sequence[a]
Ori-F	5′-CCC**GTCGAC**CGAACGCCTTGCCTTCTATCTGCG-3′
Ori-R	5′-CCC**TCTAGA**CATAATGGTGTCCGTTCTTCTATT-3′
Kan-F	5′-CCC**GAATTC**CCC**AGTACT**CCC**TCTAGA**CACGTTG TGTCTCATAATCTCTGATG-3′
Kan-R	5′-CCC**ACTAGT**TAATAAAACTCATCGAGCATCAAATGA-3′
KanP-F	5′-GGG**GTCGAC**GGGGAAAGCCACGTTGTGTCT-3′
KanP-R	5′-GGG**GGATCC**ACC*ATGATGATGATGATGATG*CATAACACCCCTTGTTA AACTGTT TATGTA-3′
RrnBT-F	5′-CCC**AGTACT**CCC**TCTAGA**TGCCTGGCGGCAGTAG CGCGGTGGTCCCAC-3′
RrnBT-R	5′-CCC**ACTAGT**AAGGCCCAGTCTTTCGACTGAGCCTTTCGT-3′
lob-2A-F	**GGATCC**ACCGTTGCCGGTGTTTCTTA
lob-2A-R	**TCTAGA**AAGAAATTGGTGGGCGACTG

[a] Restriction sites are shown in bold font. His-tag fusion sequence is shown in bold font and italics.