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Use of the Riboflavin Synthase Gene (*ribC*) as a Model for Development of an Essential Gene Disruption and Complementation System for *Haemophilus influenzae*

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We have developed a system for rapid and reliable assessment of gene essentiality in *Haemophilus influenzae* Rd strain KW20. We constructed two "suicide" complementation vectors (pASK5 and pASK6) containing 5′ and 3′ regions of the nonessential *ompP1* gene flanking a multiple cloning site and a selectable marker (a chloramphenicol resistance gene or a tetracycline resistance cassette). Transformation of *H. influenzae* with the complementation constructs directs chromosomal integration of a gene of interest into the *ompP1* locus, where the strong, constitutive *ompP1* promoter drives its expression. This single-copy, chromosome-based complementation system is useful for confirming the essentiality of disrupted genes of interest. It allows genetic analysis in a background free of interference from any upstream or downstream genetic elements and enables conclusive assignment of essentiality. We validated this system by using the riboflavin synthase gene (*ribC*), a component of the riboflavin biosynthetic pathway. Our results confirmed the essentiality of *ribC* for survival of *H. influenzae* Rd strain KW20 and demonstrated that a complementing copy of *ribC* placed under control of the *ompP1* promoter reverses the lethal phenotype of a strain with *ribC* deleted.

Haemophilus influenzae is a nonmotile, aerobic, gram-negative coccobacillus that colonizes the human upper respiratory tract as a normal commensal organism. *H. influenzae* is one of the most common agents of community-acquired pneumonia and causes a number of other diseases ranging in severity from otitis media and sinusitis to meningitis (10). Increasing emergence of virulent, antibiotic-resistant strains of *H. influenzae* and other important bacterial pathogens is driving the need for better-targeted pharmaceutical therapies (6, 7, 21).

The bacterial genomic era began in 1995, when the first complete genome of a free-living organism, *H. influenzae*, was published (5). Since then, genomics has played an increasingly significant role in the identification and validation of novel targets for antibacterial drug discovery (9). In this sense, "target validation" refers to experimental confirmation that a specific gene product is essential for the viability of an organism during growth and infection (9). Although the *H. influenzae* genome sequence was completed several years ago, the function and essentiality of many of its annotated genes remain unknown. Genome scale studies have been performed with *H. influenzae* in order to identify genes essential for growth and survival (1, 13). These studies focused on the identification of nonessential genes by performing transposon mutagenesis so that essential genes (EGs) could be deduced by mutant exclusion and zero-time analysis (1, 13). Akerley et al. (1) estimated that *H. influenzae* carries 478 EGs (no insertions) of which greater than 50% (259 genes) had no ascribed function (1). This is an efficient way to generate an inventory of potentially EGs; however, more directed studies are required to fully validate targets for antimicrobial drug discovery.

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It is critical to develop genetic tools that will allow one to conclusively define gene essentiality, as well as address questions related to physiological functions. In many cases, a gene disruption can impact neighboring genes (e.g., operon structure), leading to pleiotropic effects and false interpretations of experimental data. Therefore, it is equally important to complement the disrupted gene in *trans* with a second copy of the gene to restore, as closely as possible, the original phenotype of the strain. Here we report the development of an efficient complementation system useful in assessing gene essentiality and function in *H. influenzae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study are listed in Table 1. Chemically competent *Escherichia coli* TOP10 cells (Invitrogen Corp., Carlsbad, Calif.) were used for cloning and propagation of plasmid constructs. Cells were maintained and selected on Luria-Bertani agar and in Luria-Bertani broth in the presence of the appropriate antibiotics. Ampicillin was used at 100 μg/ml, chloramphenicol was used at 50 μg/ml, and tetracycline was used at 20 μg/ml for selection in *E. coli*. Transformation of *E. coli* cells was performed as described by the manufacturer (Invitrogen).

H. influenzae Rd strain KW20 (5, 19) was grown and maintained at 37°C on brain heart infusion agar (Difco Laboratories) supplemented with 10 μg of βNAD per ml and 12 μg of hemin per ml (sBHI; Sigma, St. Louis, Mo.) (4). *H. influenzae* transformants were selected on sBHI containing the appropriate concentration of antibiotics and other supplements. In this case, chloramphenicol was used at 1.5 μg/ml, kanamycin was used at 10 μg/ml, tetracycline was used at 5 μg/ml, and riboflavin was used at 100 μg/ml. Transformation of *H. influenzae* was performed as previously described (4, 16).

***H. influenzae* competent cell preparation and transformation.** Competent cells were prepared and transformed as previously described (4). Briefly, *H. influenzae* cells were grown overnight on sBHI agar. Riboflavin and kanamycin were added to the medium for the *ribC* KO strain. Cells were scraped from the plates, suspended in brucella broth, and used to inoculate 100 ml of sBHI broth to a starting optical density at 600 nm of about 0.07 to 0.08. Cells were grown with shaking (160 rpm) at 37°C to an optical density at 600 nm of about 0.3, at which time they were centrifuged (15 min at 3,000 × *g*), and the pellet was washed by gentle pipetting in 50 ml of freshly prepared MIV medium (4). The washed cells were centrifuged as described above, resuspended in 80 ml of MIV medium, and

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*) *7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
H. influenzae		
Rd KW20	WT genome sequence strain	19
SM1	Rd(Δ*ribC*::Kan) Kan^r	This study
ASK1	Rd(Δ*ompP1*::*cat*) Cam^r	This study
ASK2	Rd(Δ*ompP1*::*tetA tetR*) Tet^r	This study
ASK3	Rd(Δ*ompP1*::*ribC*$^+$ *cat*) Cam^r	This study
ASK4	Rd(Δ*ompP1*::*ribC*$^+$ *tetA tetR*) Tet^r	This study
ASK5	Rd(Δ*ribC*::Kan Δ*ompP1*::*ribC*$^+$ *cat*) Cam^r Kan^r	This study
SM2	Rd(Δ*ribC*::Kan Δ*ompP1*::*ribC*$^+$ *tetA tetR*) Tet^r Kan^r	This study
Plasmids		
pUC18K	Km^r Ap^r; pUC18 containing nonpolar *aph-A3* cassette	8
pUC19	Ap^r; cloning vector	20
pGEM-T	Ap^r; TA cloning vector	Promega
pCMR	Cm^r; 1.3-kb Cm^r cassette from pACYC184 in pCR2.1	18
pGESYII	Tc^r; 2.8-kb Tc^r cassette from pGJB103 in pACYC177	14
pSM1	Ap^r; pGEM-T 2,039-bp DNA fragment containing *ribC* and flanking region	This study
pSM2	Km^r Ap^r; pSM1 with 442-bp deletion in *ribC* replaced by *aph-A3*	This study
pSM3	Ap^r Tc^r; pGEM-T::*tetA tetR*	This study
pASK1	Ap^r; pGEM-T, 645-bp *ompP1* 5′ homology sequence	This study
pASK2	Ap^r; pGEM-T, 606-bp *ompP1* 3′ homology sequence	This study
pASK3	Ap^r; pUC19 with *ompP1* 5′ 3′ homology sequence	This study
pASK4	Ap^r Cm^r; pGEM-T, 1238-bp Cm^r cassette from pCMR flanked by SmaI, BamHI, XbaI, SalI, and PstI sites	This study
pASK5	Ap^r Cm^r; pUC19, *ompP1* 5′, 3′ homology sequence	This study
pASK6	Ap^r Tc^r; pUC19, *ompP1* 5′, 3′ homology sequence	This study
pASK7	Ap^r; pGEM-T::*ribC*	This study
pASK8	Ap^r Cm^r; pUC19, *ompP1* 5′, 3′ homology sequence; *ribC*	This study
pASK9	Ap^r Tc^r; pUC19, *ompP1* 5′, 3′ homology sequence; *ribC*	This study

grown with shaking (100 rpm) for 100 min at 37°C for competence development. Competent cells were frozen in 1-ml aliquots containing 20% (vol/vol) glycerol at −80°C.

One-milliliter aliquots of frozen cells were thawed on ice and transferred into 15-ml screw-cap tubes. Cells were pelleted, and each aliquot was resuspended in 1 ml of fresh MIV medium. For each transformation, approximately 1 μg of DNA was added to the cells and incubated at 37°C. After 30 min, 5 ml of sBHI broth was added to each transformation and incubation was continued for an additional 3 h. Finally, the cells were pelleted, resuspended in 100 μl of brucella broth, and plated on sBHI agar plates containing the appropriate supplements. Transformants were obtained after overnight incubation of the plates at 37°C.

Molecular biology procedures. *H. influenzae* chromosomal DNA was prepared with a Wizard genomic DNA purification kit, and plasmid DNA was prepared with a Wizard Plus plasmid miniprep kit (Promega, Madison, Wis.). Restriction

TABLE 2. Primers used for plasmid constructions

Primer	Sequence (5′→3′)
CAT1	CCCGGGGATCCTCTAGAGTCGACCTGCAGCCTCCACGGGGAGAGCCTGA
CAT2	CCCGGGGATCCTCTAGAGTCGACCTGCAGCCACCCCGTCAGTAGCTGAACAGGAGGG
CAT3	CATTGGGATATATCAACGGTGG
CAT4	GATTCAGGTTCATCATGCC
TetRF	TGCTGCAGTTAAGACCCACTTTCACATTTAAGTTGTTTTTCTAATCCGCAAATGATCAATTCAAGGCC
TetAF	TAACCCGGGAAGCTTGCATGCCTGCAGACATCATTAATTCCTAATTTTTGTTGAC
TetAR	TTGCCCGGGAAGCTTGCATGCTGCAGGCTATTTACCGCGGCTTTTTATTGAGC
ompP1	CGTACGGACGTCAAGCGTATTCAGCAAGCG
ompP2	TTTTTTCATATGGAACCCTTTGTTATAAATAAAAATCTGGG
ompP3	CCTTACCTGCAGGTATTGCTTACGATCAGGC
ompP4	CTCGCAAAGCTTGCCAAATGGCTTGTTGAAATGCG
5′ ompP1	GAAATTGAGCTACATTCAGACGG
3′ ompP1	GCTCTCCATAAGTTGAAAGCTCG
HI-1613-1F	CGAAAATTAATCAGCATCAAATAAGC
HI-1613-1R	CAAGGCGAGGCTTTAGAAATTTCGCC
HI-1613-2F	CCACAAACCCGGGCTATTGTAGATACGGTAG
HI-1613-2R	ATTTCCCGGGCTTTACGCATTTCAGGTAGTAATTTTACC
HI-1613-3F	GGTAATGACCATCGCAGGCTTGG
HI-1613-3R	CGGCTCAAATGTTATTTGCGC
K1	CTCCCACCAGCTTATATACCTTAGC
K2	TTGCCTTCTGCGTCCGGTCGATCAGG
ribCNdeF	AAAATTCATATGTTTACTGGAATTGTACAAGGC
ribCSalR	TTTTAAGTCGACCTAGAAATTTTTTGACTGCAAATAATTTTCTACCG

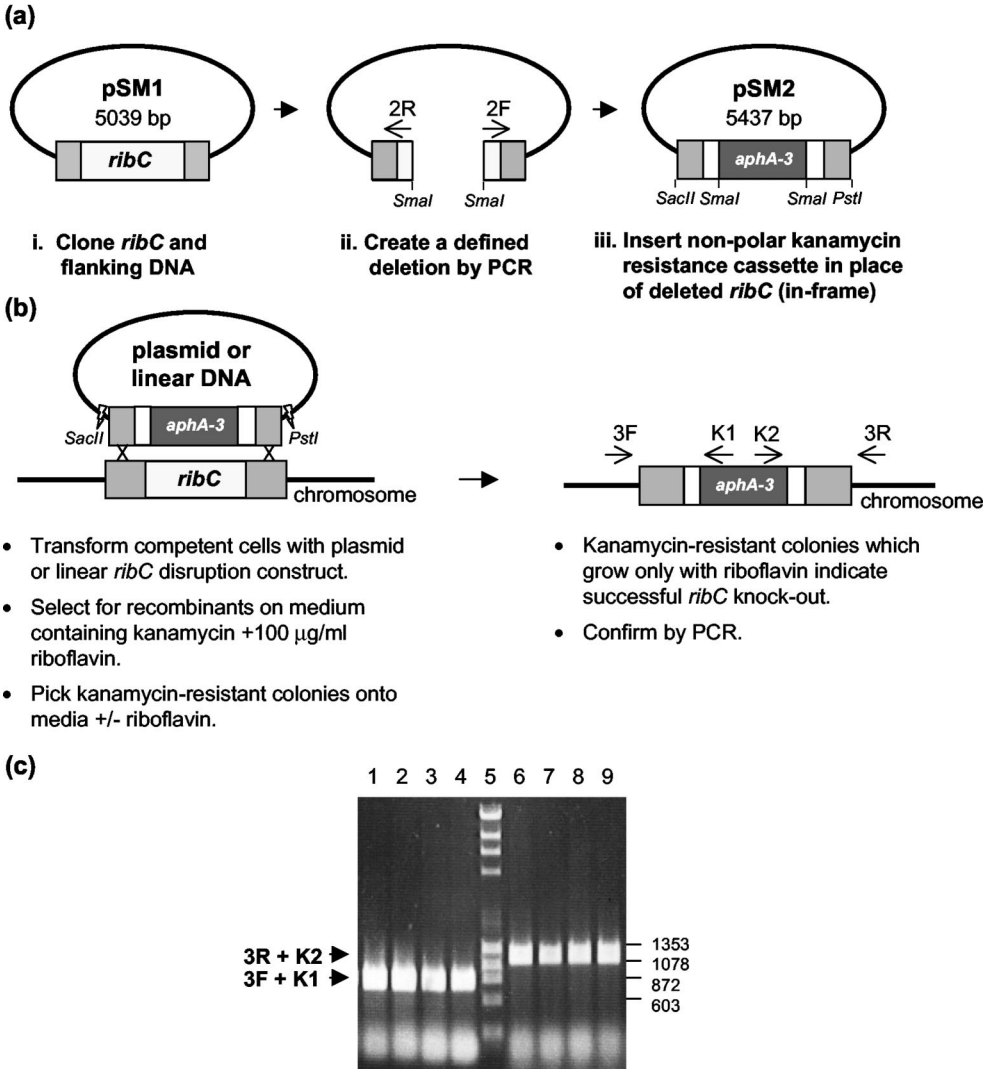

FIG. 1. (a) Construction of *ribC* disruption plasmid pSM2. (i) *ribC* (615 bp) and flanking DNA sequences (498 bp upstream of the ATG start codon and 926 bp downstream of the TAG stop codon) were cloned as a 2,039-bp PCR product into pGEM-T. (ii) A defined 442-bp deletion was created in the *ribC* coding sequence by inverse PCR with primers 2F and 2R, generating a 4,597-bp product that was then digested with SmaI. (iii) An 840-bp SmaI-digested DNA fragment containing a promoterless, nonpolar kanamycin resistance cassette (*aphA-3*) was ligated, in frame, in place of the deleted *ribC* sequence to make pSM2. (b) pSM2 or a linear *ribC* disruption construct was transformed into *H. influenzae* to generate a *ribC* disruption strain (SM1). The linear *ribC* disruption construct was generated by digestion of pSM2 with SacII and PstI, followed by gel purification. Primers 3R and K2 and primers 3F and K1 were used to verify the disruption of *ribC* by PCR amplification. 3F and 3R bind outside of the originally cloned 2,039-bp DNA fragment of pSM1 (c) PCR products obtained from four representative Kan^r colonies with primers 3R and K2 (lanes 1 to 4) and primers 3F and K1 (lanes 6 to 9) were analyzed by agarose gel electrophoresis. Only transformants grown in the presence of riboflavin carried a disrupted copy of *ribC* as determined by visualization of the expected 740-bp bands in lanes 1 to 4 (crossover at the 5′ end) and 1,195-bp bands in lanes 6 to 9 (crossover at the 3′ end). DNA size standards are shown in lane 5 with corresponding relevant sizes (base pairs) at the right.

enzymes were obtained from New England Biolabs (Beverly, Mass.). Ligations were performed with a Rapid DNA ligation kit (Roche Diagnostics Corp., Indianapolis, Ind.). PCR Supermix High Fidelity (Invitrogen) was used to generate DNA fragments, and the reactions were purified with a QuickStep PCR purification kit (Edge Biosystems, Gaithersburg, Md.). All PCR-generated clones and selected PCR-generated DNA fragments were sequenced with an ABI Prism 3100 Genetic Analyzer after preparing ABI Prism BigDye Terminator Cycle Sequencing v.2.0 Ready Reactions (PE Biosystems, Foster City, Calif.). The resulting DNA sequence chromatographs were assembled and analyzed with Sequencher software v.4.0.5 (Gene Codes Corporation, Ann Arbor, Mich.). Oligonucleotide primers used for PCR and sequencing were synthesized at Invitrogen (Table 2). DNA was extracted from preparative agarose gels with a QIAEX II kit (QIAGEN Inc., Valencia, Calif.). The cloning vectors used were pGEM-T (Promega) and pUC19 (20).

Plasmid constructions. The plasmids used in this study are listed in Table 1. To generate an *H. influenzae* riboflavin synthase (*ribC* gene number HI1613) disruption plasmid, a 2,039-bp PCR fragment containing the *ribC* gene plus 498 bp upstream of the ATG start codon and 926 bp downstream of the TAG stop codon, was PCR amplified with primers HI-1613-1F and -1R and subsequently cloned into pGEM-T to create pSM1 (Fig. 1a). Next, primers HI-1613-2F and -2R (with a SmaI site) were used to perform an inverse PCR around pSM1, creating a defined 442-bp deletion (out of 615 bp) in *ribC*. The resulting PCR product was digested with SmaI. An 840-bp DNA fragment containing a promoterless *aphA-3* kanamycin resistance cassette was excised from pUC18K with SmaI and ligated in frame in place of the 442-bp deletion in *ribC* to make pSM2 (8). In addition, a 2,039-bp linear version of the disruption construct was prepared by digesting pSM2 with PstI and SacII and gel purifying the DNA fragment containing the disrupted *ribC* gene (Fig. 1b). This plasmid and

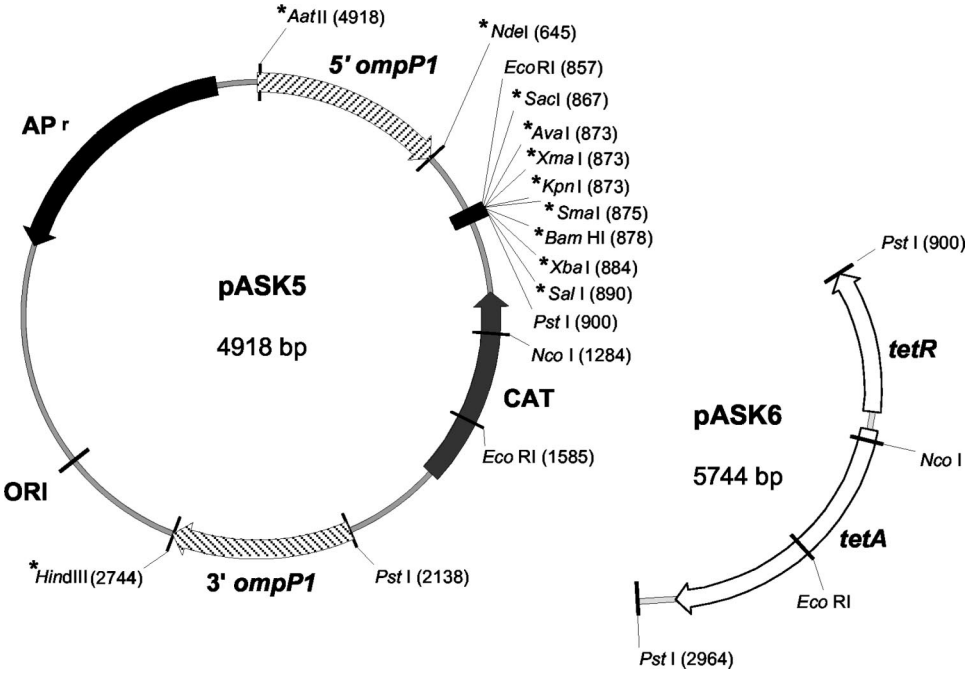

FIG. 2. Complementation vectors developed for *H. influenzae*. pUC19-based vectors pASK5 (4,918 bp) and pASK6 (5,744 bp) were constructed as described in Materials and Methods. Genomic sequences found flanking the *ompP1* coding region (645 bp upstream and 606 bp downstream) were inserted into both vectors. An NdeI site located 3′ to the 645-bp upstream *ompP1* sequence fragment allows in-frame insertion of the gene of interest. A multiple cloning site allows directional cloning. pASK5 and pASK6 contain resistance genes for chloramphenicol (*cat*) and tetracycline (*tetA* and *tetR*), respectively, to allow selection of integrants. An asterisk indicates a unique restriction enzyme site.

linear DNA fragment were used to make the strains with *ribC* disrupted that are described in Results.

Two complementation vectors were constructed to enable complementation analysis of EG disruptions in *H. influenzae*. The nonessential *ompP1* locus (HI-0401) was used as the target for homologous recombination of complementing DNA into the chromosome. First, primers ompP1 and ompP2 (containing engineered AatII and NdeI sites, respectively) were used to amplify a 645-bp region of the Rd KW20 chromosome 5′ to and including the *ompP1* start (ATG) codon (5′ *ompP1*). This fragment was cloned into pGEM-T to make pASK1. Primers ompP3 (with a PstI site) and ompP4 (with an HindIII site) were used to generate a 606-bp fragment at the 3′ end of the gene (3′ *ompP1*) that was also cloned into pGEM-T to make pASK2. The 5′ *ompP1* and 3′ *ompP1* DNAs were subsequently excised from pASK1 (AatII-NdeI fragment) and pASK2 (PstI-HindIII fragment) and directionally cloned into pUC19 to make pASK3. Both DNA fragments contained the 9-bp core DNA uptake sequence (AAGTGCGGT) required for efficient transformation of *H. influenzae* (15). Next, antibiotic resistance cassettes were amplified for cloning into pASK3. A 1,238-bp chloramphenicol resistance cassette containing the chloramphenicol acetyltransferase gene (*cat*) was amplified from pCMR (18) with the CAT1 and CAT2 primers, which were designed to introduce SmaI, BamHI, XbaI, SalI, and PstI restriction sites flanking the *cat* gene. This PCR product was cloned into pGEM-T to make pASK4. The chloramphenicol resistance cassette was then excised from pASK4 with PstI and ligated into pASK3 to make pASK5 (Fig. 2). A tetracycline resistance cassette (2,064 bp) comprising *tetA* and *tetR* was amplified with primers TetRF and TetAR from pGESYII (14) and cloned into pGEM-T to make pSM3. The cassette was modified to remove an internal NdeI site in *tetR* and incorporate flanking PstI restriction sites for subcloning. The tetracycline resistance cassette was excised from pSM3 with PstI and ligated into pASK3 to make pASK6 (Fig. 2).

The *ribC* gene (615 bp) was PCR amplified from *H. influenzae* Rd KW20 genomic DNA with primers *ribC*NdeF and *ribC*SalR and cloned into pGEM-T to make pASK7. The primers were designed so that the resultant PCR product would contain a 5′ NdeI site and a 3′ SalI site to facilitate subcloning into complementation vectors pASK5 and pASK6. *ribC* was then subcloned into both complementation vectors to make pASK8 and pASK9.

In all cases, both strands of each clone, including the junctions, were sequenced.

RESULTS

The basic strategy for complementation of EG disruptions described in this report is outlined in Fig. 3. The first step involves transformation of wild-type (WT) *H. influenzae* cells with a complementation vector carrying a functional copy of an EG and a unique antibiotic resistance gene(s) (*cat* or *tetA tetR*). We tested our complementation system with the conditionally essential *ribC* gene. *ribC* was selected to validate the complementation plasmids because a *ribC* disruption mutant is expected to be auxotrophic and therefore should be viable when the recombinant strain is grown in the presence of excess riboflavin. This allows us to study *ribC* as a nonessential gene. Additionally, when a strain with *ribC* disrupted is deprived of riboflavin, it should not be viable and would therefore allow us to study *ribC* as an EG for the sake of EG complementation.

Construction of a strain with *ribC* disrupted. Since *ribC* was our gene of choice for validating the complementation system, we first created an *H. influenzae* strain with *ribC* disrupted to verify that loss of this gene function would result in a distinguishable phenotype that could subsequently be complemented in accordance with the strategy described in Fig. 3. A schematic of the gene disruption procedure is illustrated in Fig. 1. WT *H. influenzae* strain KW20 was first transformed with *ribC* disruption plasmid pSM2, and transformants were selected on sBHI agar containing kanamycin, with and without riboflavin (Table 3). Since all of the plasmids used for transformation of *H. influenzae* in this study contain a ColE1 origin, they are unable to autonomously replicate and must integrate into the chromosome to survive antibiotic selection. After

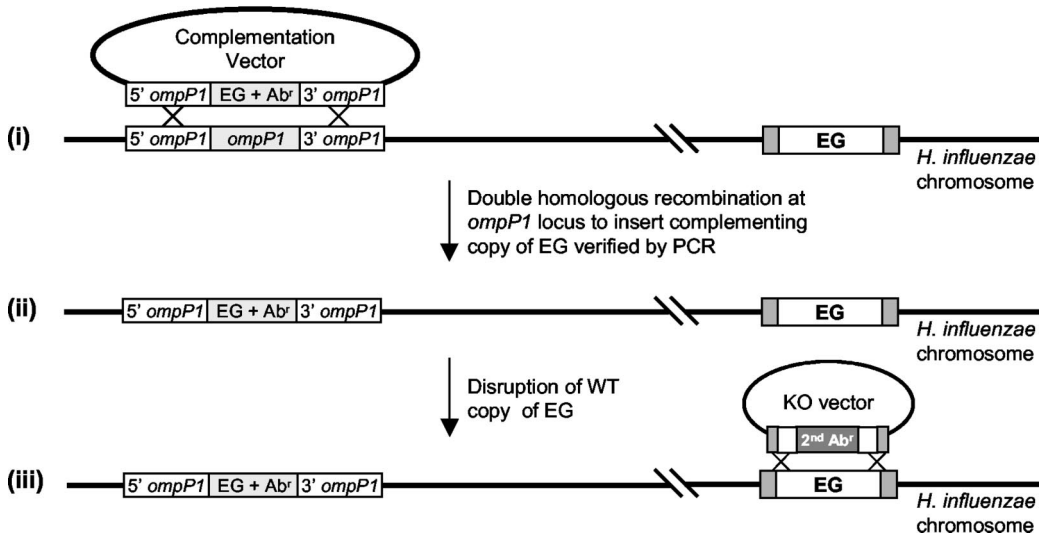

FIG. 3. Chromosomal complementation strategy for assessing gene essentiality in *H. influenzae*. (i) WT *H. influenzae* cells are transformed with a suicide complementation vector containing a copy of a putative EG together with an antibiotic resistance (Ab^r) gene for selection. Integration of this copy of the EG is targeted to the nonessential *ompP1* locus facilitated by *ompP1* 5′ and 3′ sequences flanking the EG-plus-Ab^r gene sequence. (ii) Transformants carrying the second copy of the EG, now placed under control of the *ompP1* promoter, are selected on medium containing the appropriate antibiotic. Proper integration is verified by PCR analysis. (iii) A suicide disruption vector containing the EG disrupted by a nonpolar Ab^r gene is transformed into the strain created in step ii. KO, knockout.

transformation and selection, kanamycin-resistant colonies that grew in the presence or absence of riboflavin were analyzed by genomic PCR. PCRs indicative of double homologous recombination at the *ribC* locus were prepared with primers 3F and K1 (recombination at the 5′ end of *ribC*) or 3R and K2 (recombination at the 3′ end) (Fig. 1b). When constructing an EG disruption, it is important to delete most of the EG of interest and provide substantial 5′ and 3′ flanking DNA in order to favor recombination at the original site of the EG and avoid recombination at the site of complementation.

PCR analysis of the transformants obtained in the presence of riboflavin indicated that *ribC* was disrupted because of double recombination at the *ribC* locus. The resulting size of the PCR products corresponded to those expected upon proper integration of the *ribC* disruption construct at the WT *ribC* locus (Fig. 1c). Also, when these colonies were subcultured onto medium without riboflavin they did not grow, as expected given that the *ribC* gene is essential for riboflavin synthesis. In contrast, PCR analysis of the transformants obtained in the absence of riboflavin generated only a single PCR product from either the 3′ or the 5′ PCR but not both. This result indicated that the entire plasmid, pSM2, had integrated via single homologous recombination at the 3′ end or the 5′ end of *ribC* and that the strain contained a disrupted *ribC* gene, as well as an adjacent functional WT *ribC* gene (data not shown).

Since transformation with pSM2 yielded a large number of false-positive (putatively disrupted *ribC*) colonies when *ribC* was treated as an EG (selection without riboflavin), we tested linear DNA (containing the disrupted *ribC* gene) excised from pSM2 with PstI and SacII and gel purified. Molar equivalent amounts of linear and plasmid (pSM2) DNAs were transformed into *H. influenzae*. Compared to transformation with plasmid DNA, transformation with linear DNA yielded much fewer background colonies when *ribC* was treated as an EG (selection without riboflavin; Table 3). When selection for Kan^r was done in the presence of riboflavin, similar transformation numbers were obtained for linear and circular DNAs (Table 3). Therefore, when testing the essentiality of a gene it appears that transformation with linear DNA significantly reduces potential false positives due to single-crossover recombination events (plasmid integration) since the linear DNA must integrate via double homologous recombination to yield a kanamycin-resistant phenotype. The magnitude of the difference in recovering false-positive colonies when transforming with linear versus plasmid is shown in Table 3.

Follow-up experiments revealed that the linear DNA version of the *ribC* disruption construct could be prepared by PCR amplification from pSM2 with primers 1F and 1R to achieve the same result as with restriction enzyme-generated linear DNA (data not shown).

Selection of a chromosomal site for complementation. In order to identify a nonessential gene that would provide a suitable site for integrating a second copy of a gene of interest, we reviewed the available literature for well-characterized,

TABLE 3. Transformation efficiency[a] of *ribC* disruption constructs

DNA construct[b]	With riboflavin[c]	Without riboflavin[d]	Ratio[e]
Plasmid DNA (pSM2)	4.9×10^5	7.1×10^3	69
Linear DNA	2.2×10^6	1×10^2	22,000

[a] Transformation efficiency refers to the number of kanamycin-resistant colonies recovered per microgram of DNA. These data represent a single representative (reproducible) experiment.

[b] *ribC* disruption constructs as referred to in Materials and Methods and Fig. 1b.

[c] Number of kanamycin-resistant colonies recovered when selection is carried out in the presence of 100 μg of riboflavin per ml.

[d] Number of kanamycin-resistant colonies recovered when selection is carried out in the absence of riboflavin.

[e] Ratio refers to the number of kanamycin-resistant colonies obtained in the presence of riboflavin divided by the number of kanamycin-resistant colonies obtained in the absence of riboflavin.

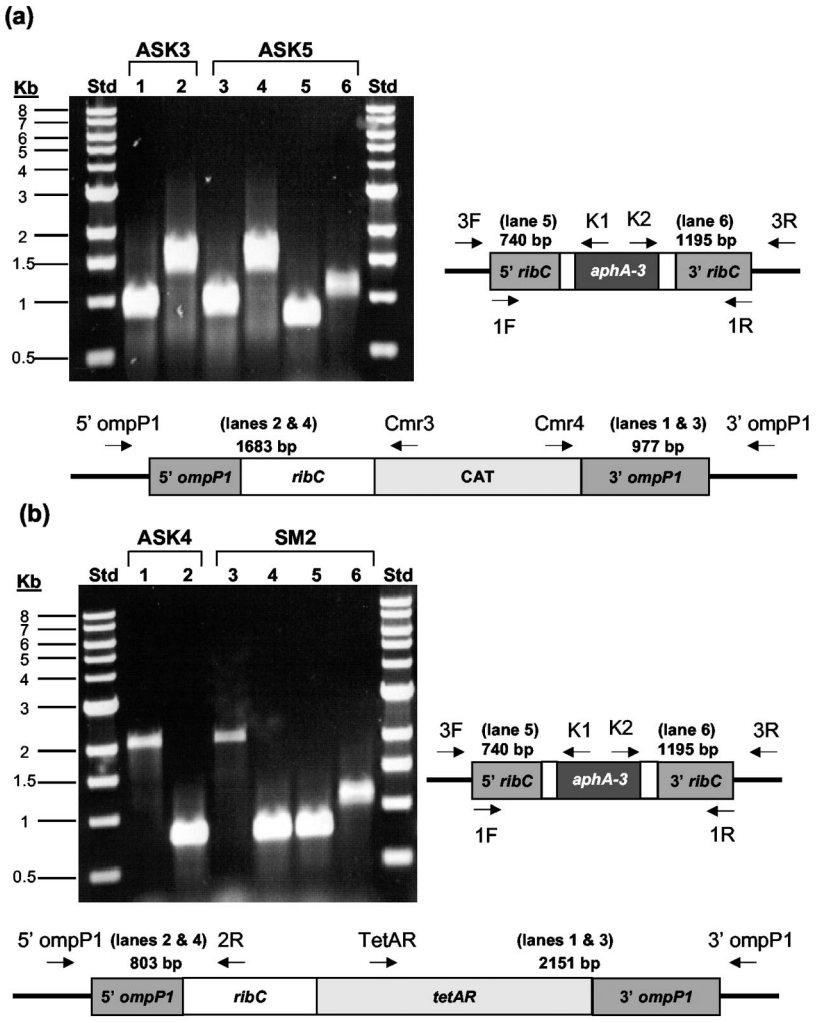

FIG. 4. Genetic complementation with complementation vectors pASK8 (a) and pASK9 (b) of strains with *ribC* disrupted. Site-specific integration of the *ribC* gene supplied on complementation vectors pASK8 and pASK9 into the *ompP1* locus to create strains ASK3 and ASK4 was verified by PCR analysis after transformation of WT strain KW20. Subsequently, targeted disruption of the authentic copy of *ribC* (but not the complementing copy at the *ompP1* locus) by transformation with pSM2 was also verified by PCR. This disruption resulted in the creation of *H. influenzae* strains ASK5 and SM2. The specific primer pairs shown in the schematics were used for PCR amplifications from chromosomal DNAs prepared from *H. influenzae* strains ASK3, ASK4, ASK5, and SM2. Schematic representations of the gene arrangements resulting from the transformations described above and the resulting agarose gel electrophoresis patterns for the PCR products obtained are shown. In lanes 1 and 3, the 977-bp ASK3 and ASK5 (a) and the 2,151-bp ASK4 and SM2 (b) products are indicative of integration of *ribC* at the 3′ end of *ompP1*, while in lanes 2 and 4, the 1,683-bp ASK3 and ASK5 (a) and the 803-bp ASK4 and SM2 (b) fragments result from recombination at the 5′ end of the *ompP1* locus. The 740- and 1,195-bp PCR products in lanes 5 and 6 of both gels verify disruption of the WT copy of the *ribC* gene. Std = size standards in kilobase pairs (Kb) are shown. Lane numbers are bracketed.

nonessential *H. influenzae* genes. We identified 16 candidates of which the outer membrane protein OmpP1 was the most suitable for our purposes. The *ompP1* gene is under control of a strong, constitutively active promoter that has been shown also to be active in *E. coli* (12). The ability to place a gene of interest under the control of this promoter would ensure robust expression in vivo, allowing effective screening in the complementation analysis. Further, the nonessential nature of this gene was confirmed by Tn*5* mutagenesis in *H. influenzae* type b and biogroup aegyptius, where OmpP1 has been extensively studied to assess its potential as a vaccine candidate (11). Strains with *ompP1* disrupted were observed to grow normally and did not exhibit any detectable phenotypic differences from the WT parent strain (11).

Complementation with pASK8 and pASK9 of an *H. influenzae* strain with *ribC* disrupted. Competent WT KW20 cells were transformed with either pASK8 or pASK9 (both carry *ribC*). Chloramphenicol (pASK8)- and tetracycline (pASK9)-resistant colonies were checked for specific homologous recombination by PCR with the primer pairs indicated in Fig. 4 and Table 2. For chloramphenicol-resistant transformants (pASK8 derived), proper integration at the 5′ and 3′ ends was verified in individual reaction mixtures containing primers 5′ompP1 and Cmr3 or primers Cmr4 and 3′ompP1, respec-

tively. Agarose gel electrophoresis of the PCR products confirmed the presence of the 977-bp (3′ end) and 1,683-bp (5′ end) fragments expected upon integration of the complementing construct at the *ompP1* locus (Fig. 4a, lanes 1 and 2). Tetracycline-resistant integrants (pASK9 derived) were similarly analyzed with primers 5′ompP1 and HI-1613-2R (5′ end) or primers TetAF and 3′ompP1 (3′ end). Recombination at both the 3′ and 5′ ends of *ompP1* was verified upon agarose gel visualization of 2,151- and 803-bp DNA fragments, respectively (Fig. 4b, lanes 1 and 2). All of the resistant colonies analyzed exhibited double homologous recombination of pASK8 and pASK9 at the *ompP1* locus. One *H. influenzae* colony each from the pASK8 (strain ASK3) and pASK9 (strain ASK4) transformations described above was subcultured, and competent cells were made for transformation with *ribC* disruption construct pSM2.

PCR-based analysis of genomic DNAs prepared from the transformants was used to verify disruption of the WT *ribC* gene. Disruption of the WT *ribC* locus was confirmed in individual PCRs set up with genomic DNAs isolated from ASK5 and SM2 (Fig. 4a and b, lanes 5 and 6). In both pASK8- and pASK9-derived strains transformed with the *ribC* disruption construct, 100% of the transformants obtained in the absence of riboflavin were confirmed as having a disrupted copy of *ribC*.

The ability of the *ribC* gene placed under control of the *ompP1* promoter to complement the *ribC* disruption was further characterized by growth on media with and without riboflavin. *H. influenzae* strain SM1 grew well on medium supplemented with riboflavin but was entirely unable to grow in the absence of riboflavin (Fig. 5, top). This conditional lethal phenotype was reversed when a complementing copy of the *ribC* gene, supplied on complementation vector pASK8 or pASK9, recombined at the nonessential *ompP1* locus, indicating that expression of *ribC* was being driven by the *ompP1* promoter (Fig. 5, bottom). *ribC* can therefore be said to be essential for *H. influenzae* because of its participation in the essential riboflavin biosynthesis pathway.

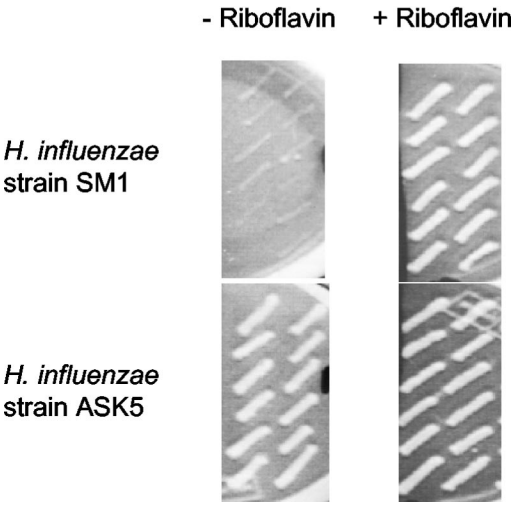

FIG. 5. Growth behavior of complemented versus noncomplemented *H. influenzae* strains with *ribC* disrupted. The growth behavior of WT *H. influenzae* cells transformed with complementation vector pASK8 and/or *ribC* disruption plasmid pSM2 was recorded in the presence and absence of riboflavin. Twelve colonies from each transformation were patched in duplicate onto media with and without riboflavin. pSM2-mediated disruption of the *ribC* gene rendered the cells dependent on an exogenous supply of riboflavin for survival (strain SM1). Introduction of a functional *ribC* gene at the *ompP1* locus as a result of transformation with the complementation vector successfully restored the WT phenotype and allowed cells to grow equally well both in the presence and in the absence of riboflavin in the growth medium (strain ASK5).

DISCUSSION

The riboflavin synthase α subunit gene, *ribC*, was identified as a model gene to test the efficiency and robustness of our system. Riboflavin, a precursor molecule of the coenzymes flavin adenine dinucleotide and flavin mononucleotide, is essential for basic cellular metabolism (2). It is produced endogenously in plants and most microbes but must be acquired exogenously by higher animals (2). Riboflavin biosynthesis has been proposed as a potential target for chemotherapy of gram-negative bacterial infections because *E. coli* and other gram-negative pathogens lack a transport system for riboflavin, and as a result, riboflavin auxotrophs only survive if grown in the presence of nonphysiologically high concentrations of the vitamin (3, 17). Little is known about riboflavin biosynthesis in *H. influenzae* other than what can be construed on the basis of homology to other tested systems.

H. influenzae is an important human pathogen. It is amenable to DNA manipulations and therefore an attractive model organism. Its genome has been completely sequenced, greatly facilitating cloning and expression of most genes of interest (5). It is naturally competent for DNA uptake and integration (15), and strain KW20 is highly sensitive to most antibiotics for which resistance cassettes are available (14, 18). The gene disruption and complementation system reported here should serve as a useful tool in evaluating gene function and essentiality in *H. influenzae*. It should find applications in the identification of genes as potential targets for drug discovery. A large number of genes can be screened, and their associated effects and functions can be studied. The use of linear versus plasmid disruption constructs greatly improves the yield of the correct double recombinants. In vivo expression of the complementing gene allows a clearer interpretation of the resultant phenotype without contending with issues of plasmid copy number, polar effects, and interference from or of other overlapping genetic elements (operon effects). We have validated the system with the conditionally essential *ribC* gene and found that our method allows efficient evaluation of putative EGs.

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