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Silencing of Essential Genes within a Highly Coordinated Operon in *Escherichia coli*

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Essential bacterial genes located within operons are particularly challenging to study independently because of coordinated gene expression and the nonviability of knockout mutants. Essentiality scores for many operon genes remain uncertain. Antisense RNA (asRNA) silencing or in-frame gene disruption of genes may help establish essentiality but can lead to polar effects on genes downstream or upstream of the target gene. Here, the *Escherichia coli* *ribF-ileS-lspA-fkpB-ispH* operon was used to evaluate the possibility of independently studying an essential gene using expressed asRNA and target gene overexpression to deregulate coupled expression. The gene requirement for growth in conditional silencing strains was determined by the relationship of target mRNA reduction with growth inhibition as the minimum transcript level required for 50% growth (MTL₅₀). Mupirocin and globomycin, the protein inhibitors of IleS and LspA, respectively, were used in sensitization assays of strains containing both asRNA-expressing and open reading frame-expressing plasmids to examine deregulation of the overlapping *ileS-lspA* genes. We found upstream and downstream polar silencing effects when either *ileS* or *lspA* was silenced, indicating coupled expression. Weighted MTL₅₀ values (means and standard deviations) of *ribF*, *ileS*, and *lspA* were 0.65 ± 0.18 , 0.64 ± 0.06 , and 0.76 ± 0.10 , respectively. However, they were not significantly different ($P = 0.71$ by weighted one-way analysis of variance). The gene requirement for *ispH* could not be determined due to insufficient growth reduction. Mupirocin and globomycin sensitization experiments indicated that *ileS-lspA* expression could not be decoupled. The results highlight the inherent challenges associated with genetic analyses of operons; however, coupling of essential genes may provide opportunities to improve RNA-silencing antimicrobials.

Many essential genes are located within operons, which can cause difficulties when studying gene functions for individual open reading frames (ORFs). *Escherichia coli* has 302 essential genes (1); 218 are in operons, of which 112 potentially are problematic due to their location (e.g., located upstream of other essential genes). Unsurprisingly, a shotgun method for creating silencers in *E. coli* found multiple essential gene silencing events arising from single expressed antisense sequences (2). In another study, certain operon genes (e.g., *eno*, *fbaA*, and *pgk*), categorized as essential for growth by knockout (negative) evidence, were not essential for growth by knockdown evidence (3). One reason for these discrepancies may be that essential gene knockouts have downstream polar effects on the expression of an intact nonessential gene. This outcome is possible for *eno*, as it is located downstream of *pyrG*, an essential gene (4). A polar effect from knockout was observed in the *ackA-pta* operon, where the disruption of *ackA* reduced Pta activity by 31% and the disruption of *pta* reduced AckA activity by 38% compared to that of the wild type (WT) (5).

Antisense silencing is a useful tool for controlling gene expression without genetic modification of the target gene. It is particularly useful for silencing essential genes because a knockdown of expression can be achieved, maintaining cell viability so long as sufficient levels of the silenced mRNA remain available for translation. Plasmids designed to express a short antisense sequence, called expressed antisense RNA (asRNA), are inexpensive to produce, do not suffer from delivery problems, can be conditionally controlled by different promoters (6, 7), and can be expanded easily to target a large number of genes (2, 8). Due to the complex-

ities of operon gene silencing, the use of expressed asRNA has been limited largely to monocistronic genes. When expressed asRNA is applied to an operon, there is often no way to ensure that only the intended target ORF is affected. One study on the *ackA-pta* operon of *E. coli* showed downstream and upstream polar effects when either gene was silenced (5), while another study on the *sol* operon of *Clostridium acetobutylicum* showed upstream polarity effects by reduced gene product levels (9). The use of antisense peptide nucleic acids (PNA) for silencing of genes in operons suffers similar problems; the *lac* operon in *E. coli* and the *cmeABC* operon in *Campylobacter jejuni* showed downstream polarity through reduced transcript or protein levels (10, 11).

To evaluate the possibility of independently silencing operon genes by previously established methods (12), we used the *E. coli*

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TABLE 1 Primer sequences and qPCR efficiencies

Primer	Sequence ^a	Target gene	Purpose and/or details ^b
Q/rpoA_F1	CCGAGGTTGAGATTGATGGT	<i>rpoA</i>	qPCR; 500 nM F1, 300 nM R1, E = 104%
Q/rpoA_R1	CTTCCTGAACGCCTTCTTTG		
Q/ribF_F1	GCAGAGTGTGGCGTTGATTA	<i>ribF</i>	qPCR; 500 nM F1, 400 nM R1, E = 102%
Q/ribF_R1	CACCAGAAGATCGCTGATGA		
Q/ileS_F	ACAAAACGCCGATCATCTTC	<i>ileS</i>	qPCR; 400 nM F, 500 nM R, E = 102%
Q/ileS_R	ACTGCACGCCTTTGATCTCT		
Q/lspA_F	GCGGCGTTTAGTTTCCTTG	<i>lspA</i>	qPCR; 400 nM F, 500 nM R, E = 102%
Q/lspA_R	AATCGCAATACCGCAAAG		
Q/ispH_F1	CATCTGTACGCCACGACTA	<i>ispH</i>	qPCR; 400 nM F1, 500 nM R1, E = 101%
Q/ispH_R1	AACACAACCTCCGCCTGTTC		
Q/16S_F2	TGCATCTGATACTGGCAAAGC	<i>rrsA</i>	qPCR; 400 nM F2, 500 nM R2, E = 97%
Q/16S_R2	ACCTGAGCGTCAGTCTTCGT		
Q/plsC_F	CCGTTCAAGACTGGAGCATT	<i>plsC</i>	qPCR; 500 nM F, 500 nM R, E = 100%
Q/plsC_R	GAGACGCACACGGGAATAAT		
Q/polA_F	CGGCAACGGTGATTTCTTAT	<i>polA</i>	qPCR; 400 nM F, 500 nM R, E = 99%
Q/polA_R	CTTTTCCAGCTTCGCAATC		
Q/mreD_F	AATGTGGGCACAGGTTTGT	<i>mreD</i>	qPCR; 500 nM F, 300 nM R, E = 104%
Q/mreD_R	GCCACCAGGTAAGCAATGAT		
Q/zipA_F	ATAAACCGAAGCGCAAAGAA	<i>zipA</i>	qPCR; 400 nM F, 300 nM R, E = 100%
Q/zipA_R	CCGCTTGTGAATGCTGTTA		
Q/fldA_F	GACATTGCAAAAAGCAGCAA	<i>fldA</i>	qPCR; 400 nM F, 300 nM R, E = 100.6%
Q_fldA_R	ACCAGTTTGCCGTTGAAATC		
AS_ribF-F4	ACACCATGGCCCTTCTTGCGGGCCTG	<i>ribF</i> (-50 to +50)	Antisense construct
AS_ribF-R4	AACTCGAGGACCGCTGTACAAGGTATACTCGGA		
AS_ileS-F1	GGACACCATGGGATCGCCACGCATCG	<i>ileS</i> (-59 to +61)	
AS_ileS-R1	TGGAACCTCGAGTAACAAAACCGGCTTAAGC		
AS_lspA-F2	GGACACCATGGCGACTACCACCAGCCAC	<i>lspA</i> (-45 to +55)	
AS_lspA-R2	TGGAACCTCGAGTGTACAGCAACGTCGC		
AS_ispH-F1	GGACACCATGGCCGTAATGGCCAGCG	<i>ispH</i> (-49 to +89)	Antisense construct
AS_ispH-R1	TGGAACCTCGAGCATTTTGATATTGAAGTGCTGG		
pHN1257_3751F	GCATAATTCGTGTCGCTCAA	pHN1257	Screening transformants
pBAD_F	ATGCCATAGCATTTTATCC	pBAD	
pBAD-R	TCTGATTTAATCTGTATCAGG		
pBAD18SacI_R	GTAGAGCTCTTCTCTCTGCTAGCCCAA	p1G2	Removal of <i>gfp</i> , insertion of SacI and XhoI in p1G2 for cloning of ORFs
pBAD18XhoI_F	TGTCTCGAGGATGATGAGTCGACCTGCA		
ribF_FSacI	TAAGAGCTCATGAAGCTGATACGGC	<i>ribF</i>	Construction of p1G2RF
ribF_RXhoI	CTACTCGAGTTAAGCCGTTTTGTTAG		
ileS_FSacI	TAAGAGCTCATGAGTGACTATAAATCAACC	<i>ileS</i>	Construction of p1G2IS
ileS_RXhoI	CTACTCGAGTCAGGCAAACCTTACGT		
lspA_FSacI	TAAGAGCTCATGAGTCAATCGATCTGTTC	<i>lspA</i>	Construction of p1G2LA
lspA_RXhoI	CTACTCGAGTTAATGTTTTTTCGCTCTAGAAG		
ispH_FSacI	TAAGAGCTCATGCAGATCCTGTTGGC	<i>ispH</i>	Construction of p1G2IH
ispH_RXhoI	CTACTCGAGTTAATCGACTTCACGAATATCG		

^a Underlined sequences indicate restriction endonuclease sites.

^b E is the percent amplification efficiency.

ribF-ileS-lspA-fkpB-ispH operon (NC_000913.3). This operon was chosen because it contains four genes (*ribF*, *ileS*, *lspA*, and *ispH*) thought to be essential for growth by knockout evidence, and each is involved in a different biological/biochemical pathway; hence, the four genes seem unlikely to regulate one another. The gene *ribF* encodes an enzyme needed for the synthesis of essential metabolites flavin mononucleotide (FMN)/flavin adenine dinucleotide (FAD), *ileS* encodes isoleucyl-tRNA synthetase, required for translation, *lspA* encodes prolipoprotein signal peptidase, required for cell wall maturation, and *ispH* encodes an enzyme needed for isoprenoid biosynthesis (13). In addition, transcriptional units for these genes are known (14–16), there is transposon-mediated knockout (negative) evidence of the essen-

tiality for the four genes (1, 17), and *ileS-lspA* overlap by one nucleotide. Finally, there are inhibitors for *IleS* (18, 19) and *LspA* (20), enabling us to assay for sensitization after silencing.

MATERIALS AND METHODS

Construction of conditional silencing strains. Antisense RNA sequences were expressed from pHN1257, an expression vector containing a kanamycin resistance gene, an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, P_{trc} , and a multiple cloning site (MCS) flanked by inverted repeats, termed paired termini, for stabilizing an inserted antisense sequence (5). Antisense sequences of 100 to 160 bases around the ribosome-binding site (RBS) and coding regions of target genes that have minimal predicted secondary structures by M-fold were chosen and amplified with appropriate primers using MG1655 genomic DNA (gDNA) as

TABLE 2 Plasmids and bacteria in this study

Strain/plasmid	Relevant feature(s)	Function	Reference or source
<i>E. coli</i> strains			
MG1655	F ⁻ , λ ⁻ , <i>rph-1</i>	Expression, PCR	CGSC, Yale University
DH5α	<i>fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	Subcloning	New England Biolabs
Plasmids			
pHN1257	pSC101H ori, Kan ^r , <i>lacI^q</i> , P _{trc} <i>lac^o</i> -PT-MCS	asRNA expression vector	5
pHNRF4	pHN1257 carrying <i>ribF</i> asRNA targeting -50 to +50 of <i>ribF</i>	IPTG-inducible asRNA expression	This study
pHNIS	pHN1257 carrying <i>ileS</i> asRNA targeting -59 to +61 of <i>ileS</i>		
pHNLA2	pHN1257 carrying <i>lspA</i> asRNA targeting -45 to +55 of <i>lspA</i>		
pHNLA3	pHN1257 carrying <i>lspA</i> asRNA targeting +1 to +100 of <i>lspA</i>		
pHNLA4	pHN1257 carrying <i>lspA</i> asRNA targeting +21 to +138 of <i>lspA</i>		
pHNLA5	pHN1257 carrying <i>lspA</i> asRNA targeting +51 to +151 of <i>lspA</i>		
pHNIH	pHN1257 carrying <i>ispH</i> asRNA targeting -49 to +89 of <i>ispH</i>		
p1G2	pBR322/ColE ori, Cm ^r , P _{BAD} , AraC N6I, V65G, L133M, E165G, E169V, C280*	ORF expression vector	23
p1G2RF	p1G2 carrying <i>ribF</i>	L-Arabinose-inducible ORF expression	This study
p1G2IS	p1G2 carrying <i>ileS</i>		
p1G2LA	p1G2 carrying <i>lspA</i>		
p1G2IH	p1G2 carrying <i>ispH</i>		

the template (Table 1). MG1655 gDNA was extracted with a GenElute bacterial genomic kit (Sigma). PCR was carried out using Phusion high-fidelity PCR master mix with HF buffer (New England BioLabs). Amplicons of the expected sizes were column purified using a MinElute PCR purification kit (Qiagen) and digested with NcoI and XhoI (Fermentas), followed by ligation to similarly digested pHN1257. Ligation reactions were transformed to DH5α cells (New England BioLabs), plated on LB-Miller broth supplemented with kanamycin (50 μg/ml; Sigma), and screened by colony PCR using a plasmid-specific forward primer, pHN1257_3751F, and an insert-specific reverse primer (e.g., AS_ribF-R4) (Table 1). Colony PCR was carried out using Crimson Taq DNA polymerase (New England BioLabs). Positive DH5α transformants were used for plasmid preparations (plasmid minikit; Qiagen) of asRNA-expressing plasmids pHNRF4, pHNIS, pHNLA2, pHNLA3, pHNLA4, pHNLA5, and pHNIH (Table 2), which were sequenced to verify the cloning of inserts and transformed into MG1655 to obtain conditional silencing strains.

Construction of ORF-expressing plasmids. Target gene overexpression was driven by the arabinose-inducible promoter P_{BAD}. An arabinose-inducible plasmid was considered, because overexpression of some essential genes is toxic to cells unless they are tightly controlled and expressed at low levels (21, 22). However, IPTG prevents efficient expression from the arabinose-inducible promoter P_{BAD}. To improve compatibility, we used a plasmid derived from pBAD with a mutated AraC (N6I, V65G, L133M, E165G, E169V, and C280* [where * represents a stop codon]), kindly provided by Jay Keasling (Table 2), shown to improve expression in the presence of IPTG (23). The *gfp* in this plasmid was removed by inverse PCR using primers pBAD18SacI_R and pBAD18XhoI_F (Table 1) and Phusion high-fidelity PCR master mix. Inserts of *ribF*, *lspA*, *ileS*, and *ispH* were amplified from MG1655 gDNA with gene-specific forward and reverse primers containing SacI and XhoI 5' ends, respectively (Table 1). Amplicons of p1G2 and ORFs were column purified (MinElute PCR purification kit; Qiagen), digested with SacI and XhoI, ligated with T4 DNA ligase, transformed to chemically competent DH5α, and selected on LB-Miller agar supplemented with chloramphenicol (30 μg/ml). Recombinant plasmids were sequenced to verify cloning of inserts.

Construction of rescue strains. Rescue strains each contain a pair of asRNA-expressing plasmid (pHNRF4, pHNIS, pHNLA2, and pHNIH in Table 2) and ORF-expressing plasmid (p1G2RF, p1G2IS, p1G2LA,

p1G2IH in Table 2). Conditional silencing strains were made competent by CaCl₂ treatment (24) for transformation of ORF-expressing plasmids and selected on LB-Miller agar supplemented with kanamycin (50 μg/ml; Sigma) and chloramphenicol (30 μg/ml; Sigma). Positive transformants were confirmed by PCR using two primer pairs: pHN1257_3751F and either ASribF-R4, ASileS-R1, ASlspA-R2, or ASispH-R1 to detect asRNA and pBAD_F/pBAD_R (Table 1) to detect ORFs.

Bacterial growth for induction of asRNA and/or ORF expression. Overnight cultures were grown at 37°C, with shaking at 180 rpm for 16 to 18 h. Cultures were standardized to 5 × 10⁵ CFU/ml by the measurement of the optical density at 600 nm (OD₆₀₀) of a 1:10 dilution and adjustment to an OD₆₀₀ value of 0.003 in LB-Miller broth supplemented with appropriate antibiotics (25). Standardized cultures were added in 180-μl volumes to each well of a 96-well plate. For induction of asRNA expression, conditional silencing strains were treated with different IPTG concentrations in 20 μl per well and incubated in a microplate reader (SpectraMax; Molecular Diagnostics) at 37°C for 5 h. Growth was monitored every 5 min after shaking for 15 s. For induction of asRNA expression and ORF expression, rescue strains were treated with IPTG and L-arabinose in 20 μl per well. The IPTG and L-arabinose combination resulting in growth rescue was determined for each rescue strain by titration of IPTG causing growth inhibition against 0.008, 0.016, 0.032, 0.064, 0.125, 0.25, and 0.5 mM L-arabinose. Plates were incubated in the microplate reader at 37°C for 18 h, and growth was monitored every 15 min after shaking for 10 s.

Bacterial growth for RNA extraction, cDNA synthesis, and qPCR. Conditional silencing strains were induced with IPTG as described above. The growth of cultures was monitored by OD₅₅₀ readings every 5 min after shaking for 5 s in a microplate reader (SpectraMax 340PC; Molecular Diagnostics), and all cultures were harvested when the untreated culture increased to an OD₅₅₀ of ≈0.1. OD₅₅₀ readings of cultures in the time period with the same IPTG treatment were used to calculate an average representative growth rate (ΔOD/Δt). Cultures with the same IPTG treatment but grown in different wells of a 96-well plate were pooled for total RNA extraction and DNase I treatment (RiboPure bacterium kit; Life Technologies). RNA was checked for gDNA contamination by qPCR using 16S primers (Table 1) and SsoFast EvaGreen supermix (Bio-Rad) before cDNA synthesis of 100 ng RNA (iScript reverse transcription supermix; Bio-Rad). Samples of cDNA were diluted 10-fold, and 4 μl was

used in each qPCR. Nontemplate controls were included for each primer pair, and technical duplicates for each sample were done in each run.

Microarray data analysis for reference genes in qPCR. Genes with the least variable expression under IPTG induction were sought in the microarray data obtained from the GEO database (26) under accession number GSE17505 (27). In addition to using the MAS5-analyzed data set, microarray data also were normalized under RMA and MAS5 methods within the affy package (28). The probe sets were ranked in order of increasing standard deviations (SD) across the whole data set for each data subset. The probe sets with the lowest average ranking had the least variation and were considered the most invariant. Seven candidate reference genes were chosen from the two lists for validation of expression stability under experimental conditions.

Validation of primers for qPCR. Primer pairs for qPCR were designed by Primer3 with a melting temperature (T_m) of 60°C and product sizes of 100 to 200 bp (Table 1). Primer concentrations (300 to 500 nM) were optimized for use in SsoFast EvaGreen supermix (Bio-Rad) in a CFX96 real-time PCR detection system (CFX Manager v2; Bio-Rad) with 10 ng MG1655 gDNA per reaction mixture. Primer efficiency percentages were determined using 10-fold serial dilutions of MG1655 gDNA (10 pg to 1 µg), and only primers with efficiencies within 5% of one another were selected for use (Table 1). Melting-curve analyses were carried out for all reactions.

Validation of reference genes and analysis of mRNA levels by RT-qPCR. The following samples were tested for candidate reference gene stability with the indicated IPTG concentrations: MG1655 containing pHN1257 induced with IPTG (0, 0.03, 0.1, 0.4, and 0.8 mM), MG1655 containing pHNIS (0 and 0.03 mM), MG1655 containing pHNLA2 (0 and 0.06 mM), and MG1655 containing pHNIH (0 and 0.8 mM). Total RNA and cDNA were prepared as described above. Raw quantification cycle (Cq) values were analyzed in qbase+ (Biogazelle, Belgium), and the expression stability (M value) of each candidate reference gene, *rrs*, *mreD*, *fldA*, *plsC*, *zipA*, *polA*, and *ispB*, was calculated as the average pairwise variation for a gene with all other tested genes (29). The lower the M value, the more stable the expression. Stepwise exclusion of the gene with the highest M value allows ranking of the tested genes according to their expression stability, and from this the optimal number of reference genes may be calculated (29). To determine the expression of genes conditionally silenced by asRNA, Cq values were normalized against the two most stably expressed reference genes with user-defined PCR efficiencies (Table 1) in qbase+ (Biogazelle). Relative mRNA levels then were calculated as induced normalized Cq divided by uninduced normalized Cq.

Determination of MTL₅₀ values and statistical analysis. Relative reductions in growth rate were plotted against relative reductions in mRNA over the same range of IPTG concentrations for each experimental repeat in Prism 6 and fitted to a four-parameter dose-response curve used for determining effective concentration inhibiting growth by 50% (EC₅₀). In this instance, the minimum transcript level required for 50% growth (MTL₅₀) was determined. Both the x and y values were variable in each experiment, and a weighted mean and standard error method was used to account for variability. The weighted MTL₅₀ means, standard deviations, and standard errors were calculated using the formulae shown below, where X_w is weighted mean, SDw is weighted SD, SEw is weighted standard error, A is the MTL₅₀ of one experiment, a is the standard error of A , and n is the number of experimental repeats.

$$X_w = \frac{(A/a^2) + (B/b^2) + (C/c^2)}{(1/a^2) + (1/b^2) + (1/c^2)} \quad (1)$$

$$\text{SDw} = \sqrt{\frac{(1/a^2)(A - X_w)^2 + (1/b^2)(B - X_w)^2 + (1/c^2)(C - X_w)^2}{[(n-1)/n][(1/a^2) + (1/b^2) + (1/c^2)]}} \quad (2)$$

$$\text{SEw} = \frac{\text{SDw}}{\sqrt{n}} \quad (3)$$

Weighted one-way analysis of variance (ANOVA) was carried out using weighted means and weighted standard errors.

MIC and growth sensitization assays. Stock solutions of synthetic globomycin (30) were dissolved in dimethyl sulfoxide (DMSO) (5.3 mg/ml), and mupirocin (AppliChem) was dissolved in ethanol (50 mg/ml). To determine MIC values, 2-fold serial dilutions of antibiotic stocks (2 to 256 µg/ml) were made in LB-Miller broth and tested for bacterial growth inhibition. The solvents DMSO and ethanol also were included in the assay to ensure they were not inhibiting growth. Bacterial cultures were prepared as described above, and 180 µl was added to 20 µl of antibiotics per well. Plates were incubated at 37°C for 24 h, and growth was visually scored.

Downregulated expression of a drug target gene results in bacterial cell sensitization to the protein inhibitor (2). This mechanism has been used to validate the mechanism of action of antibacterials (2, 31) and was applied here to validate gene specificity of asRNA silencers. Antibiotic sensitization of conditional silencing strains was carried out by titration of either globomycin or mupirocin (0, 0.01, 0.1, 1, 2, 4, 8, 16, and 32 µg/ml) against IPTG concentrations effective for growth inhibition in a checkerboard format before the addition of 180 µl bacterial culture to a final volume of 200 µl. Plates were incubated at 37°C for 24 h, and growth was visually scored. Where there was sensitization in the presence of IPTG, the assay was repeated twice more, with the lowest IPTG concentration resulting in this phenotype. Antibiotic sensitization of rescue strains pHNIS/p1G2LA and pHNLA2/p1G2IS was carried out at 0.03 mM and 0.1 mM IPTG, respectively, with or without 0.03 mM L-arabinose, in the presence of globomycin and mupirocin as described above. The combination of IPTG and L-arabinose was determined in rescue assays as described above.

RESULTS

Silencing of *ribF*, *ileS*, *lspA*, and *ispH* was gene specific and revealed different requirement stringencies. To test the specificity of expressed asRNA against the essential genes *ribF*, *ileS*, *lspA*, and *ispH*, conditional silencing strains were induced with IPTG and growth was monitored. Growth inhibition was observed for expressed asRNA against *ileS*, *lspA*, and *ribF* but not *ispH* (see Fig. S1 in the supplemental material). We then tested whether the conditional silencing strains could be sensitized to relevant protein inhibitors and whether the growth inhibition due to gene silencing could be rescued by target gene overexpression. Previously we showed that overexpression of a gene targeted by expressed asRNA resulted in rescue from growth inhibition (12); therefore, it may provide a way of compensating for polar silencing effects. Mupirocin inhibits the isoleucyl-tRNA synthetase encoded by *ileS* (19), and globomycin inhibits the prolipoprotein signal peptidase II encoded by *lspA* (20). There are no known protein inhibitors of RibF and IspH. Both *ileS*- and *lspA*-silencing strains were sensitized to both mupirocin and globomycin with induction of asRNA, while the *ribF*- and *ispH*-silencing strains did not change in susceptibility to globomycin or mupirocin with induction of asRNA (Table 3). As *ileS* and *lspA* overlap by one nucleotide, it was possible that silencing of either gene resulted in polar silencing of the other, and sensitization to both protein inhibitors of LspA and IleS was observed.

Another method for determining silencer specificity is by overexpressing the gene target to supply functional protein to a level that would counteract the effects of silencing. Logically, the reverse also would indicate the specificity of a silencer in cases where gene overexpression is toxic, since depletion of a chromosomal gene product by silencing could equilibrate levels of the overexpressed gene to that in a normal cell. This method is useful when

TABLE 3 Sensitization of conditional silencing strains to mupirocin and globomycin

Conditional silencer	Target gene	Fold reduction in MIC ^a of:	
		Mupirocin	Globomycin
pHNR4	<i>ribF</i>	0	0
pHNIS	<i>ileS</i>	8 ^b	2 ^b
pHNLA2	<i>lspA</i>	2 ^c	2 ^c
pHNIH	<i>ispH</i>	0	0

^a Fold reduction relative to the level for uninduced cultures from three experiments. The MIC of mupirocin and globomycin was 16 μ g/ml in uninduced conditional silencing strains.

^b At 0.03 mM IPTG.

^c At 0.1 mM IPTG.

protein inhibitors are lacking, as is the case for RibF and IspH. Growth inhibition due to overexpression of *ribF* in rescue strains was greater than growth inhibition due to silencing of either gene (Fig. 1b), and growth was rescued with expression of asRNA at 0.8 mM IPTG and the corresponding ORF at 0.125 mM L-arabinose, indicating that the expressed antisense silencer of *ribF* was gene specific (Fig. 1b). Although growth of the empty plasmid control strain was reduced with the addition of 0.125 mM L-ara (Fig. 1c), growth of the *ribF* rescue strains at 0.8 mM IPTG and 0.125 mM L-ara was similar to that of uninduced cultures. Growth inhibition due to silencing of *ileS*, *lspA*, or *ispH* in rescue strains was greater than growth inhibition due to overexpression of the target genes compared to levels for uninduced cultures and control strains

(Fig. 1b and c). However, growth was not rescued with expression of asRNA and ORF for the *ileS* and *lspA* strains, suggesting either the silencers were not gene specific or the two genes were tightly coupled. As growth inhibition due to *ispH* silencing was weak, rescue in growth was weak (Fig. 1b and c).

We then analyzed target mRNA reduction along with growth inhibition. In order to accurately normalize gene expression to that of reference genes, we selected seven genes displaying stable expression based on microarray data and determined gene expression stability in 11 samples (see Table S1 in the supplemental material). The optimal combination of stably expressed genes was *zipA* and *rrs*, which were used for normalization of qPCR data. Three IPTG concentrations of up to 1 mM, resulting in a reduction of growth rate, were chosen for each silencing strain, and the corresponding mRNA reduction of target genes was determined. Reductions in *ribF* and *ispH* mRNA were greater than reductions in growth rates, particularly in *ispH*, where only a 24% reduction in growth rate was achieved with 43% reduction in mRNA (Fig. 2). In contrast, reductions in *ileS* and *lspA* mRNA were less than reductions in growth rates at the two highest inducer concentrations tested (Fig. 2).

Log₁₀ values (mRNA reduction) then were plotted against values for relative growth reduction as a dose-response curve to enable calculation of differences in gene requirement for growth, indicated by the MTL₅₀ (12). This value measures the minimum level of transcript required to sustain 50% cell viability, so that a value approaching 0 indicates no requirement (e.g., nonessential

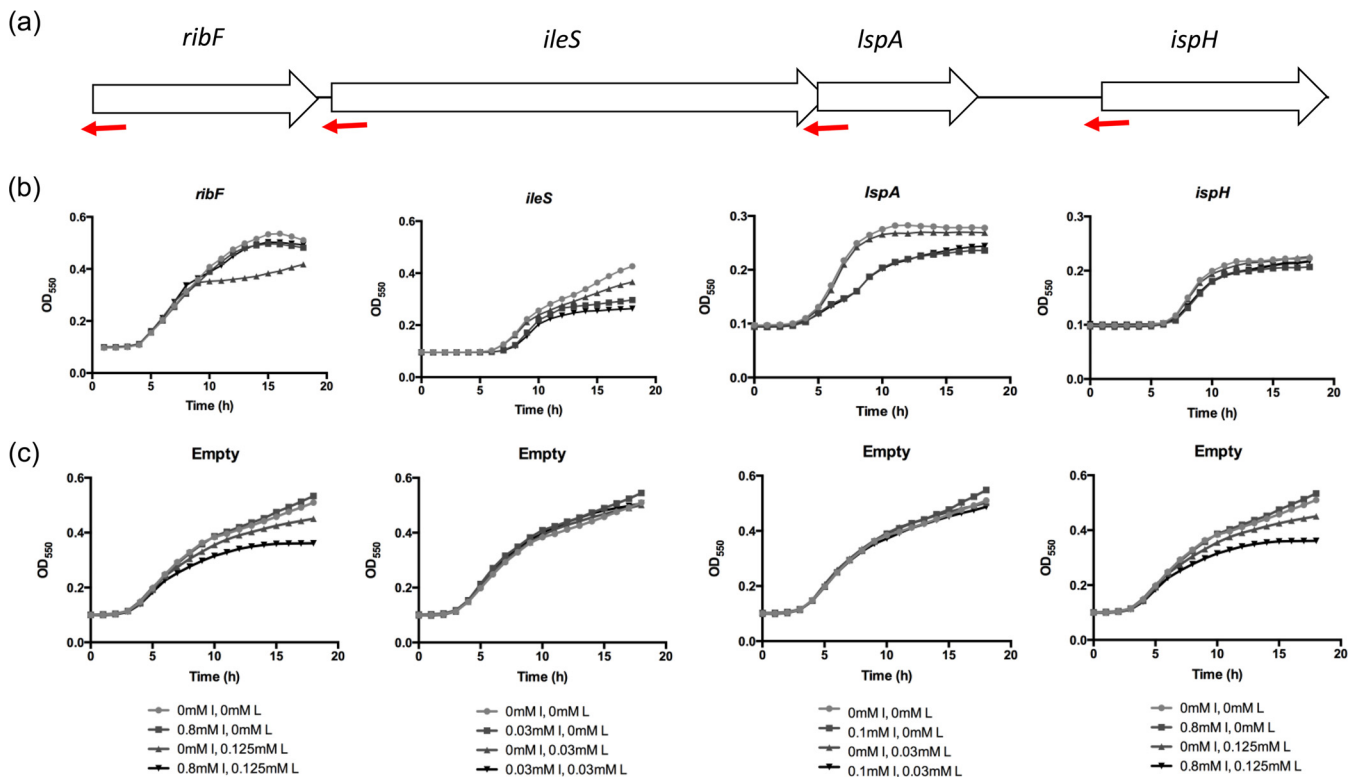


FIG 1 Validation of asRNA silencing specificity through overexpression of targeted ORF in rescue strains. (a) Gene organization of the operon, with asRNA complementary regions in red. (b) Growth of rescue strains induced with IPTG (I) for asRNA expression and L-arabinose (L) for ORF expression. (c) Growth of control rescue strains containing empty pIG2 plasmid at the same inducer concentrations as those used for panel b. Concentrations of IPTG (I) and L-arabinose (L) are indicated. Mean 18-h growth curves are plotted ($n = 3$).

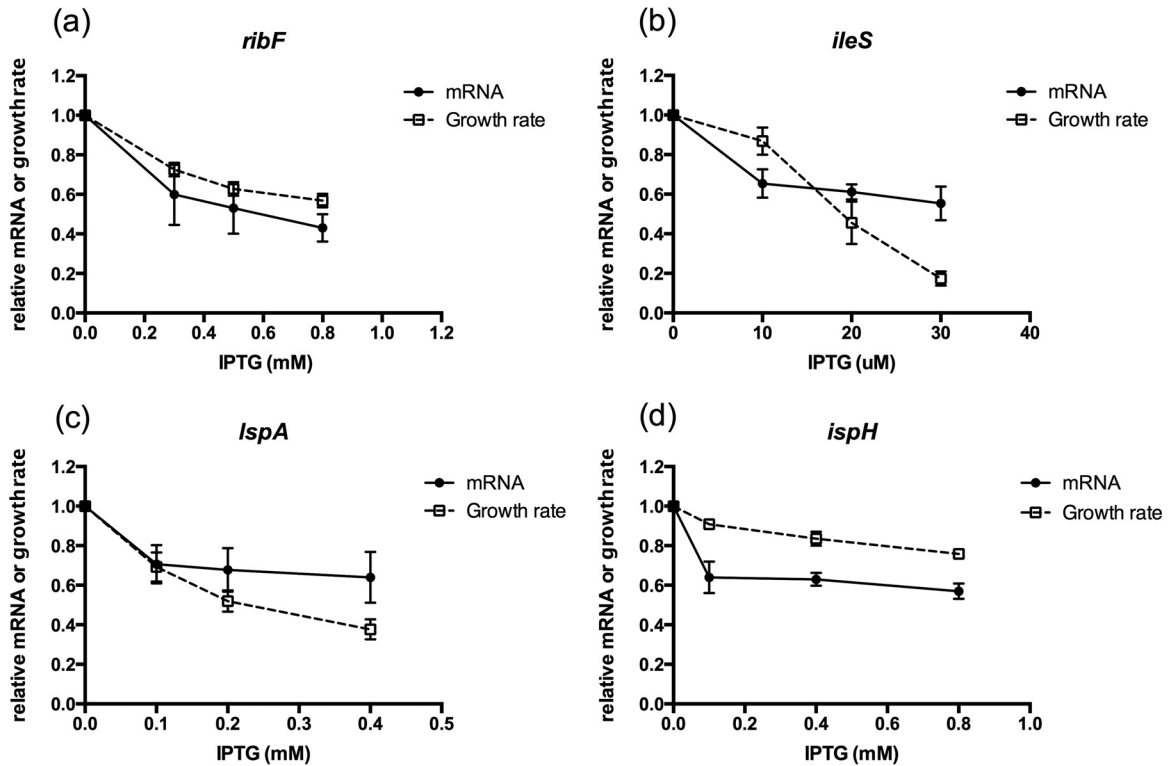


FIG 2 Bacterial growth rate ($\Delta OD/\Delta t$) and mRNA reductions with IPTG induction of expressed antisense-targeting essential genes. Growth rate and mRNA reductions were calculated relative to those of uninduced cultures (averages \pm SD; $n = 3$).

gene) and a value approaching 1 indicates high requirement for viability. For this purpose, an additional IPTG dose (0.04 mM) for maximum inhibitory response was used for *ileS*. Similar to determining EC_{50} s from a dose-response curve, the mean weighted MTL_{50} of *ribF*, *ileS*, and *lspA* were calculated as 0.65 ± 0.18 , 0.64 ± 0.06 , and 0.76 ± 0.10 , respectively (Fig. 3). However, the MTL_{50} values were not significantly different ($P = 0.71$ by weighted one-way ANOVA). MTL_{50} for *ispH* could not be determined, as growth reductions were not sufficient for good curve fits in two of three experiments.

Silencing of *ileS* or *lspA* results in polar effects. We next ex-

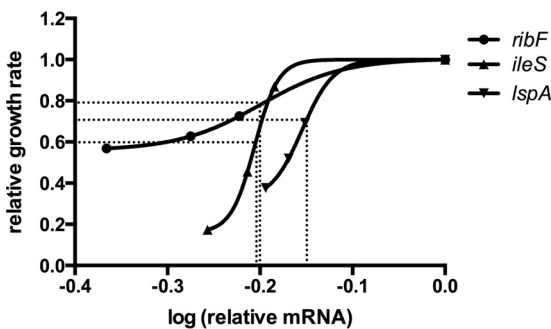


FIG 3 Use of mRNA and growth rate covariation to determine the MTL_{50} of essential genes *ribF*, *ileS*, and *lspA*. Transformed mean values of relative mRNA levels were plotted against mean values of the relative growth rate shown in Fig. 2 ($n = 3$). Dotted lines indicate the minimum transcript level value of each gene (x axis) required to support 50% of maximal (uninduced) bacterial growth (y axis). Curves and MTL_{50} were generated in GraphPad Prism 6.

amined polar silencing effects for each silencer by measuring the mRNA levels of all genes in the operon upon silencer induction. The *ribF* and *ispH* silencers did not result in polar silencing, indicating that independent gene silencing in this operon is possible (Fig. 4). However, *ileS* asRNA induction significantly reduced downstream transcripts of *lspA*, and *lspA* asRNA induction significantly reduced upstream transcripts of *ileS* (Fig. 4). These results may explain the earlier observation of cross-sensitization of both *ileS* and *lspA* silencing strains to mupirocin and globomycin (Table 3) and the lack of growth rescue when asRNA and ORFs were expressed together (Fig. 1b).

To examine whether we could silence *lspA* without an upstream effect on *ileS*, we created three more expressed antisense plasmids, pHNLA3, pHNLA4, and pHNLA5 (Table 2), avoiding the *ileS* ORF while targeting the coding gene of *lspA*. The expression levels of all four essential genes in the operon were determined from three experimental repeats, and each silencing strain was tested for sensitization to globomycin (Table 4). Results indicated that silencing of *lspA* was coordinated with *ileS*, and silencers targeting regions further into the coding region (i.e., pHNLA4 and pHNLA5) were not effective compared to silencers targeting regions at the start of the coding region (i.e., pHNLA2 and pHNLA3) (32). We were not able to create asRNAs that silenced *lspA* or *ileS*; hence, their expression had to be decoupled by compensating for the effects of the nontargeted gene.

Polar silencing effects of *ileS* and *lspA* cannot be decoupled. The *ileS* gene is transcribed in two transcriptional units, while *lspA* is transcribed in three transcriptional units (Fig. 5a). To decouple the silencing polarity of *ileS* and *lspA*, overexpression of either

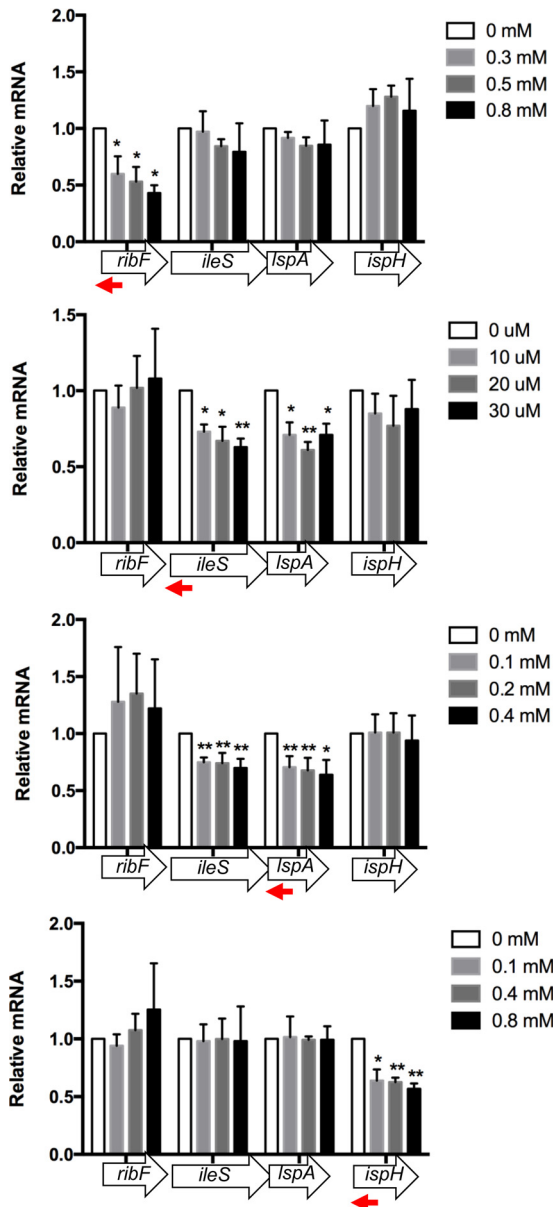


FIG 4 Expression profiles of each essential gene within the *ribF-ileS-lspA-fkpB-ispH* operon upon silencing one essential gene. Target regions for expressed antisense sequences specific for individual target genes are indicated by red arrows. Averages \pm SD of mRNA levels relative to those of the uninduced control are shown ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

gene should result in a phenotype representative of the uncompleted, silenced gene. That is, previously observed cross-sensitivity to mupirocin or globomycin shown in Table 3 should be reduced or eliminated. For an *ileS*-specific silencing phenotype, a strain containing *ileS* asRNA and *lspA* overexpression (pHNIS/p1G2LA) (Fig. 5b) was made for antibiotic sensitization assays with 0.03 mM IPTG for induction of *ileS* antisense and either with 0.03 mM L-arabinose or without L-arabinose for induction of *lspA* expression. The IPTG and L-arabinose concentrations used here were the same as those for conditional silencing strains (Table 3) and rescue strains (Fig. 1). The pHNIS/p1G2LA strain had MICs of 8 μ g/ml and 32 μ g/ml for mupirocin and globomycin, respec-

TABLE 4 Efficacy of asRNA silencers targeting *lspA*

Antisense plasmid	Target region of <i>lspA</i>	Growth inhibition with IPTG	Reduction in mRNA level ^a				Fold reduction in globomycin MIC ^b
			<i>ribF</i>	<i>ileS</i>	<i>lspA</i>	<i>ispH</i>	
pHNLA2	-45 to +55	Yes	-	+	+	-	4
pHNLA3	+1 to +100	Yes	-	+	+	-	4
pHNLA4	+21 to +138	Yes (slight)	-	-	-	-	0
pHNLA5	+51 to +151	Yes (slight)	-	-	-	-	0

^a Only mRNA significantly reduced ($n = 3$; $P < 0.05$) in a dose-dependent manner are considered. -, no reduction; +, reduction.

^b Fold reduction in MIC at 0.2 mM IPTG was determined relative to the level of uninduced cultures in two repeated assays. Growth was scored visually.

tively, whereas the conditional silencing strain had a MIC of 16 μ g/ml to both antibiotics under noninduced conditions (Table 3). With *ileS* asRNA expression under IPTG induction, the range of fold reduction in mupirocin MICs with *lspA* expression under L-arabinose induction was similar to that of cultures lacking L-arabinose (Table 5). With *ileS* asRNA expression under IPTG induction, the reduction in globomycin MIC also was the same with and without *lspA* expression under L-arabinose induction (Table 5). Therefore, simultaneous expression of *lspA* and antisense sequence against *ileS* did not improve sensitization to mupirocin over globomycin. However, the pHNIS/p1G2LA strain displayed lower mupirocin MICs and higher globomycin MICs than the pHNIS conditional silencing strain.

To test for a phenotype specific for silencing of *lspA*, a strain containing *lspA* asRNA and *ileS* overexpression (i.e., pHNLA2/p1G2IS) (Fig. 5c) was constructed and used in antibiotic sensitization experiments as described above, except 0.01 mM IPTG was used as the most effective induction concentration. In contrast to the pHNIS/p1G2LA strain, antibiotic MICs of the pHNLA2/p1G2IS strain remained the same as those for the conditional silencing strain of *lspA* under noninduced conditions (i.e., 16 μ g/ml) (Table 3). With *lspA* asRNA expression induced under IPTG,

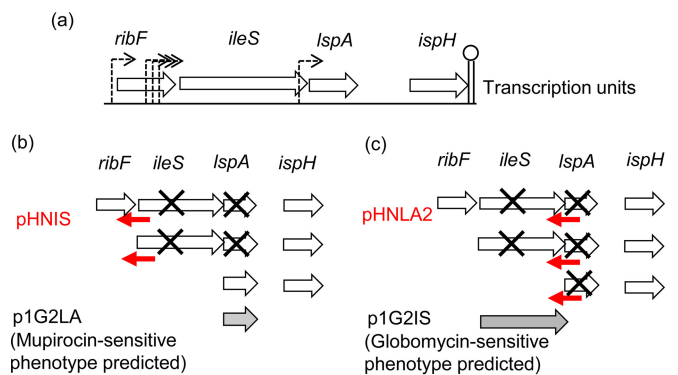


FIG 5 Designs for decoupling *iles-lspA* expression by asRNA silencing and nontarget gene expression. (a) Known transcriptional units of the *ribF-ileS-lspA-fkpB-ispH* operon showing only the essential genes (14–16). Dashed arrows indicate promoter sites. (b) The *ileS* silencer, pHNIS (red arrow), silences *ileS* and *lspA* (crosses) on two of three possible transcriptional units. For an *ileS*-specific response with pHNIS, *lspA* should be expressed from p1G2LA (gray arrow) to compensate for loss of *lspA* expression. This is predicted to result in a mupirocin-sensitive phenotype. (c) The *lspA* silencer, pHNLA2 (red arrow), silences *ileS* and *lspA* (crosses) on all possible transcriptional units. For an *lspA*-specific response from pHNLA2, *ileS* should be expressed from p1G2IS (gray arrow) to compensate for loss of *ileS* expression. This is predicted to result in a globomycin-specific phenotype.

TABLE 5 Sensitization of strains expressing asRNA and nontarget ORF to mupirocin and globomycin

Conditional silencer	Gene silenced (mM IPTG)	ORF expressed (mM L-ara)	Fold reduction in MIC of:			
			Mupirocin		Globomycin	
			0 mM L-ara	0.03 mM L-ara	0 mM L-ara	0.03 mM L-ara
pHNIS/p1G2LA ^a	<i>ileS</i> (0.03)	<i>lspA</i> (0.03)	8–16	8–32	4–8	4–8
pHNLA2/p1G2IS ^b	<i>lspA</i> (0.1)	<i>ileS</i> (0.03)	4	4	4	4

^a MICs of mupirocin and globomycin were 8 µg/ml and 32 µg/ml, respectively, without IPTG and L-arabinose (L-ara) induction.

^b MIC of mupirocin and globomycin was 16 µg/ml without IPTG and L-ara induction.

^c Fold reduction relative to the level of uninduced asRNA cultures from three experiments.

the fold reductions in mupirocin and globomycin MICs were the same with and without *ileS* expression induced under L-arabinose (Table 5). There was no reduction in cross-sensitization to mupirocin, indicating uncoupling of *ileS* from *lspA* was not achieved with simultaneous expression of *ileS* and antisense sequence against *lspA*.

DISCUSSION

For the *E. coli ribF-ileS-lspA-fkpB-ispH* operon, we determined gene requirement stringencies for bacterial growth and the effect of silencing one gene on the gene expression of the rest of the operon. Although three promoters of *ileS* were identified within *ribF* (33, 34), silencing of *ribF* did not lead to downstream polar effects on the expression of *ileS*. This suggests an advantage of using asRNA over disruption or deletion of *ribF*, which could remove *ileS* promoter sites and result in downstream inhibition of *ileS* transcription. Two studies were not able to knock out *ribF* (*yaaC*) (1, 17), but one study did (35), leaving uncertainty about its requirement. Our results support *ribF* as an essential gene.

The MTL₅₀ value for *lspA* was not significantly greater than that for *ileS* and *ribF*; however, other studies have scored the gene requirement of *lspA* as greater than that of *ileS* in terms of the viability of knockout mutants in rich and minimal media (1). It is possible that growth in minimal media enhances differences in MTL₅₀ values. Indeed, we observed greater growth inhibition by pHNRF4 in M9 minimal media than in LB (unpublished data). Insufficient growth reduction from the *ispH* silencer may be due to silencer design, although up to a 43% reduction in *ispH* transcripts was achieved. Alternatively, it may show that 57% of normal levels of transcript and/or enzyme was sufficient to support growth in a rich medium. A study examining the depletion of *ispH* (*lytB*) under arabinose induction in a mutated *ispH* strain found cells were able to grow for about 4 h before cell death (36). It is probable that *ispH* requirement for growth is the lowest of the four essential genes examined under the tested conditions.

Upstream silencing in an operon through expression of asRNA does not appear to be limited to overlapping genes. In creating a genome-wide library of *E. coli* silencers, upstream silencing effects were shown indirectly in the *rpsF-priB-rpsR-rplI* operon, where a silencer against a nonessential gene (*rplI*) resulted in growth inhibition, presumably due to a polar silencing effect on the upstream, nonoverlapping, and essential gene, *rpsR*, that was 41 nucleotides away (2). A study by Tummala et al. applied similar tools to two nonoverlapping, nonessential genes in the tricistronic *aad-ctfA-ctfB* operon in *C. acetobutylicum* (9). Silencing of *ctfB* reduced CtfA and CtfB levels (9); *ctfA* and *ctfB* are one nucleotide apart.

Therefore, proximity and transcription units may be factors in upstream polar silencing, since it did not occur for *ribF* when *ileS* was targeted or for *lspA* when *ispH* was targeted. Naturally occurring antisense small noncoding RNAs (sRNA) have been reported to repress genes downstream of the targeted ORF. Two examples are sRNAs of the *phoPQ* operon in *E. coli* (37) and the YPK_1206-1205 operon in *Yersinia pseudotuberculosis* (38), where sRNAs silence the target and downstream genes. It will be interesting to see if upstream silencing exists in nature as more sRNAs are identified and characterized.

We considered the possible effects of pHNLA2 on *ileS* due to gene overlap with *lspA* and created silencers targeting deeper into the *lspA* coding sequence to avoid nonspecific effects on *ileS*. However, these silencers were less efficient, possibly because they are not likely to repress translation initiation. Many known mRNA degradation mechanisms are centered on the RBS or initiated by activities occurring around the RBS (39). For instance, bacterial mRNA is vulnerable to degradation by RNases through direct entry (40) when ribosome and RNAP are physically separated or uncoupled by translation repression (41). Another RBS-centered mechanism is activation of mRNA cleavage to occur at a site distal from mRNA-asRNA binding at the RBS (42). Therefore, while a silencer targeting the RBS is more effective, it may result in nonspecific activity in complex overlapping gene targets, such as *iles-lspA*.

Cross-sensitization of *ileS* and *lspA*-silencing strains to globomycin and mupirocin could not be reduced by complementation, indicating the complexity in expression and regulation of the two overlapping essential genes. Systemic physiological effects of translation errors from silencing *ileS* and cell wall weakening from silencing *lspA* may add to the difficulty of reducing cross-sensitization. An *E. coli* mutant with defective IleS was shown to increase antibiotic susceptibility to mupirocin and other antibiotics because of the cumulative global effects of incorrect codon translation (43). Inhibition of LspA by globomycin leads to the accumulation of prolipoprotein within the periplasm and cell death (44, 45), presumably as a result of the loss in cell wall structure and integrity (46). It is possible that cells simultaneously expressing *ileS* and antisense against *lspA* suffered from cell wall malformation and increased permeability to mupirocin without depletion of IleS.

In summary, this study shows essential genes in an operon may have different requirements for growth in rich media, and it was not possible to decouple expression of overlapping *ileS* and *lspA* genes using a combination of expressed antisense and ORF expression vectors due to inherent complexities in gene regulation. However, this strategy enabled the examination of essential gene

associations within an operon and is a powerful tool for prokaryotic genetics. Overlapping putative essential genes in operons identified by knockout techniques may need closer examination prior to accurate scoring of gene function and requirements. Potentially, such gene structures can be used to our advantage as gene targets for antimicrobials.

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