Table 2. Cross-resistance phenotype for genes enriched by Plas-Seq

Strain	MIC (μg ml^{-1})				
	CRO*,†	GEN*,†	LEV*,†	TET†,‡	TMP*,†
TOP10	0.13	0.50	0.016	1.00	0.25
pFF6§	0.13	0.50	0.016	1.00	0.25
ftsI‖	0.50	0.50	0.016	1.00	0.25
marC‖	0.25	1.00	0.031	2.00	0.50
marC‖ *(antisense)*	0.13	0.50	0.016	1.00	0.25
marAB‖	0.25	0.50	0.031¶	2.00¶	0.25
ampC‖	0.25	0.50	0.016	1.00	0.25
rob‖	0.25	0.50	0.031	2.00	0.25
nlpE‖	0.25	1.00	0.031	2.00	0.25
yebV‖	0.25	1.00	0.031¶	1.00	0.25
soxS‖	0.25¶	0.50	0.031	2.00	0.50¶
sdiA‖	0.25	0.50	0.016	2.00	0.50¶
folA‖	0.13	0.50	0.031	1.00	4.00

*MIC measured by agar dilution.

†Genes selected with specific antibiotics are underlined.

‡MIC measured by macrodilution.

§The pFF6 vector (KAN resistance marker) was use as a control since the pZErO-2 plasmid (KAN resistance marker) is a suicidal vector.

‖Gene expressed in pZErO-2 plasmid.

¶These genes were enriched <eightfold in these samples and at early selection steps.

noticeable effect on the MIC of GEN, CRO or LEV. To test whether this was general or specific to *yebV*, we also targeted the transcriptional factor *rob*. Instead of using a gene knockout strategy, we used the CRISPR interference (CRISPRi) technique to knock-down the expression of *rob*. CRISPRi uses a catalytically dead Cas9 along with a guide RNA which interfere with transcription in bacterial and eukaryotic cells [15, 19]. As verified by quantitative RT-PCR, we successfully knocked down the expression of *rob* when a perfect match guide RNA was used but not when the guide had two mismatches at its 3′ end just upstream of the protospacer adjacent motif (PAM) (Fig. 3). In contrast to *yebV*, cells with less *rob* mRNA had a phenotype and were more susceptible to CRO (Fig. 3).

DISCUSSION

We have adapted a functional gene overexpression screen coupled to NGS to *E. coli*. This allowed the rapid screening of five antibiotics and pinpointing of possible targets and genes decreasing susceptibility. Obviously, considerable work has already been done with *E. coli* and the five drugs tested but, nonetheless, we identified a role for a new gene, *yebV*. Several other enriched plasmids were not tested (Table S3), and it is possible that genes never before associated with resistance could indeed contribute to this phenotype. The overexpression of *yebV* led to reduced susceptibility not only to GEN but also to CRO and LEV (Table 2). The phenotype is only seen upon overexpression as the knock-out of *yebV* (Fig. 2) did not lead to increased drug sensitivity. Further work is warranted for understanding how YebV influences susceptibility to antibiotics.

Plas-Seq led to the isolation of drug targets including FtsI for CRO and FolA for TMP. Mutations in *ftsI* have been associated with increased resistance to β-lactam antibiotics in *Haemophilus* spp. [20], and the accumulation of mutations in *ftsI* can lead to CRO resistance [21]. While mutations have been described, overexpression of *ftsI* as a resistance mechanism to CRO seems to be novel. Overexpression of the dihydrofolate reductase gene *folA* was already known to contribute to TMP resistance [22]. It is salient to point out that *folA* overexpression led to the highest level of resistance (16×) and isolation of this gene using overexpression strategies seems to be frequent using a number of antifolates [4, 8]. The outer membrane lipoprotein NlpE was isolated with both CRO and GEN screens (Table 1). NplE is a known activator of systems involved in cell wall homeostasis, and its overexpression was shown to activate multidrug efflux pumps in *E. coli* leading to a decreased susceptibility to various antibiotics [23]. NplE, however, does not seem to confer a phenotype in all genetic backgrounds as exemplified here for ATCC 25922 (Table 1). Similarly, overexpression of *sidA* increases the MIC to several antibiotics by regulating the AcrAB effux pump [24] but this is the first time to our knowledge that it has been associated with TET and cross-resistance to CRO (Table 2), a phenotype consistent with *sidA* overexpression observed in *E. coli* cells resistant to ceftazidine [25].