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Inhibition of the Multiple Antibiotic Resistance (*mar*) Operon in *Escherichia coli* by Antisense DNA Analogs

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The multiple antibiotic resistance operon (*marORAB*) in *Escherichia coli* controls intrinsic susceptibility and resistance to multiple, structurally different antibiotics and other noxious agents. A plasmid construct with *marA* cloned in the antisense direction reduced LacZ expression from a constitutively expressed *marA*::*lacZ* translational fusion and inhibited the induced expression of LacZ in cells bearing the wild-type repressed fusion. The *marA* antisense construction also decreased the multiple antibiotic resistance of a Mar mutant. Two antisense phosphorothioate oligonucleotides, one targeted to *marO* and the other targeted to *marA* of the *mar* operon, introduced by heat shock or electroporation reduced LacZ expression in the strain having the *marA*::*lacZ* fusion. One antisense oligonucleotide, tested against a Mar mutant of *E. coli* ML308-225, increased the bactericidal activity of norfloxacin. These studies demonstrate the efficacy of exogenously delivered antisense oligonucleotides targeted to the *marRAB* operon in inhibiting expression of this chromosomal regulatory locus.

Although bacterial antibiotic resistance has been recognized since the advent of antimicrobial agents, the consequences of the emergence of resistant microorganisms were historically controlled by the continued availability of effective alternative drugs. Today, the situation is rapidly changing. Drug resistance has emerged as a serious medical problem in both hospitals and the community, leading to increasing morbidity and mortality (25). The problem is worsened by the growing number of pathogens resistant to multiple, structurally unrelated drugs and by the fact that no new class of antimicrobials is likely to be introduced before the end of the decade (25).

The multiple antibiotic resistance (*mar*) locus controls the expression of chromosomal genes involved in intrinsic multidrug susceptibility and resistance in *Escherichia coli* and other members of the *Enterobacteriaceae* (5–8, 10, 12, 13). In the presence of selective agents, like tetracycline or chloramphenicol, Mar mutants arise spontaneously at a frequency of 10^{-7} (12). The *mar* locus is composed of two transcriptional units, *marC* and *marRAB*, that are divergently transcribed from a central regulatory region, *marO* (7, 14) (Fig. 1). MarR negatively regulates the expression of the *marRAB* operon; overexpression of MarA, a transcriptional activator, leads to increased resistance to multiple drugs by activating or depressing a number of genetic loci in *E. coli* that contribute to the Mar phenotype (7, 10, 18). The function of *marC* is yet unknown.

Transcription of the *marRAB* operon can be increased three- to sixfold by tetracycline, chloramphenicol, salicylate, and other compounds (7, 8, 34). When activated, cells become resistant not only to multiple antibiotics but also to oxidative stress agents and organic solvents (2–4, 7, 15, 36). Besides being resistant to multiple drugs (e.g., tetracycline, chloramphenicol, rifampin, penicillins, and cephalosporins), Mar mutants are protected from the killing effects of fluoroquinolones (14). Mutations in the *mar* locus appear to increase the chances of accumulating secondary mutations leading to the expression of higher levels of resistance to antimicrobial agents (5, 12). Mar mutants have been found among fluoroquinolone-resistant clinical isolates of *E. coli* (20).

Antisense-oligonucleotide technology has been shown to be an effective means of controlling gene expression in infectious disease agents associated with overproduction of specific gene products (11, 19, 27, 29–31). Relatively few studies have examined the use of antisense oligonucleotides to inhibit gene expression in bacteria (11, 19, 31). The present experiments were conducted to determine the feasibility of introducing antisense oligonucleotides to control expression of the *mar* locus in *E. coli* so as to make the bacteria more susceptible to antimicrobial agents associated with the Mar phenotype.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study and their relevant properties are listed in Table 1. Unless otherwise noted, bacteria were grown and maintained at 30°C in Luria-Bertani (LB) broth or on LB agar plates containing the appropriate antibiotics for selection. *E. coli* ML308-225 may possess permeability characteristics that facilitate passage of DNA analogs through its membrane (19, 28).

Construction of chromosomal *marORA*::*lacZ* translational fusions. A translational-fusion plasmid (pMLB1034) was used for constructing *marA*::*lacZ* fusions bearing mutant and wild-type *marR*. An 818-bp *Dra*I fragment, containing mutant or wild-type *marR* (*marOR* and 144-bp of *marA*), was cloned into the *Sma*I site of pMLB1034. The resulting plasmids, pKMN14 and pKMN18, have the *marA* fusion in the same translation frame with *lacZ*. The *marOR*A* fragment of pKMN14 was derived from the plasmid pHHM191 (7, 16), which has a missense mutation (asterisk) at the 45th amino acid of the sequence encoded by *marR* (*marR2*). pKMN18 contained wild-type *marOR(A)* cloned from plasmid pHHM183 (16).

The *marOR*A*::*lacZ* and *marORA*::*lacZ* translational fusions in plasmids pKMN14 and pKMN18 were transduced via a λ site-specific recombination mechanism into the chromosome of ASS111, which contains a 1.24-kb *marORAB* deletion and is Δ*lacZ*, Δ*phoA*, and *recA* (33). First, the fusion plasmids were introduced by transformation individually into ASS110, a *recA*$^+$ strain (33). The transformants were infected with λRZ5 to allow the formation of λRZ5 (*marORA*::*lacZ*) recombinants. The recombinant lysate was used to transduce

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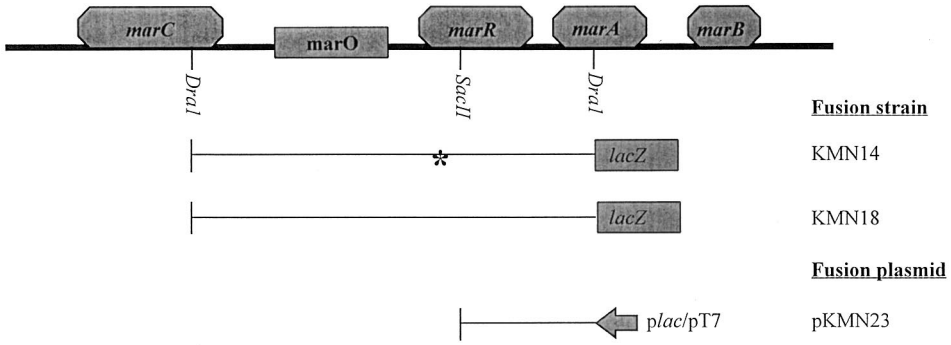

FIG. 1. Diagram of the *E. coli mar* locus showing two divergently transcribed units, *marC* and *marRAB*. The *marORA*::*lacZ* chromosomal translational-fusion strains KMN14 and KMN18 were constructed with an 818-bp *Dra*I fragment from either wild-type pHHM183 (KMN18) or pHHM191, which has a missense mutation (asterisk) at the 45th amino acid of MarR (KMN14); both fragments were cloned in frame with *lacZ* in pMLB1034. The antisense *marA* fusion plasmid pKMN23 was created with a 473-bp fragment generated through a *Sac*II-*Dra*I digest of pHHM183 cloned in the antisense direction behind the *lac* promoter-T7 polymerase system in pSPOK.

the plasmidless strain ASS110, and ampicillin-resistant lysogens were selected on ampicillin (50 μg/ml). Lysates from these purified lysogens were then used to infect ASS111.

The presence of the *marA*::*lacZ* fusion in the chromosomal DNA of ampicillin-resistant lysogens was confirmed by PCR. Primer RK1, 5′-GTGAAAAGTACC AGCGATCTG-3′, which can hybridize to the 5′-terminal end of *marR*, and primer LZ, 5′-ATGTGCTGCAAGGCGAT-3′, which can anneal to the internal portion of *lacZ*, were used for *marOR*::*lacZ* fragment amplification.

Cloning of *marR* and antisense *marA* under the control of the *lac* and T7 promoters. Primer ORAB2, 5′-GGACTGCAGGCTAGCCTTGCATCGCAT-3′, can hybridize with nucleotides 1311 to 1328 in *marO* (GenBank accession no. M96235) and create a *Pst*I site. Primer RK3, 5′-TCTTGAATTCTTACGGCA GGACTTTCTTAAG-3′, can hybridize with nucleotides 1858 to 1879 at the 3′-terminal end of *marR* and create an *Eco*RI site. These primers were used for amplification of the *marOR* fragment from the wild-type *E. coli* strain AG100 and its Mar mutant, AG102. The resulting 570-bp *Pst*I-*Eco*RI PCR fragments were cloned into the *Pst*I-*Eco*RI site of the plasmid pSPOK, a kanamycin resistance derivative of pSPORT1 (Gibco/BRL) (20). The *marOR*-carrying pSPOK plasmids (pSPOK-*marR* and pSPOK-*marR1*) can be induced for expression of the wild-type or mutant *marR* genes from the *lac* promoter by isopropyl-β-D-thiogalactopyranoside (IPTG) or from the T7 promoter by T7 RNA polymerase.

A 473-bp fragment (330 bp of *marR* and 143 bp of *marA*) from the *Sac*II site in *marR* to the *Dra*I site in *marA* of pHHM183 was inserted into the *Sna*BI site of pSPOK. The resulting construct, pKMN23, contained part of the *marRA* sequence in the orientation opposite that of the *lac* promoter in pSPOK (antisense).

Cloning of *marA* and *marORAB*. Based on the known DNA sequence of the *mar* locus (GenBank accession no. M96235), PCR primers which flanked the coding sequence and allowed amplification of *marA* and the *marORAB* regions were created; these amplified regions were then cloned behind the T7 promoter of pBluescript KS (Stratagene). The *marA* PCR primers were designed to amplify the *marA* coding sequence from bp 1893 to 2282, resulting in a 389-bp product. A *marORAB* PCR product (1,281 bp) was created as well with primers designed to amplify the DNA sequence of *marORAB* from bp 1311 to 2592 of the published sequence (7). Restriction endonuclease sites for *Eco*RI and *Pst*I were incorporated into the ends of PCR primers to ensure that the inserted fragments were in the correct orientation when cloned into pBluescript, creating pDW10 (pBluescript-*marA*) and pDW11 (pBluescript-*marORAB*). To ensure that the proper DNA fragments were cloned, the DNA sequences of the cloned PCR

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description or characteristic	Reference or source
E. coli strains		
AG100	Wild-type strain	12
AG102	Mar mutant of AG100	12
ASS110	*recA*$^+$ parental strain of ASS111	33
ASS111	ASS110 bearing a 1.24-kb *marORAB* deletion, Δ*lacZ*, Δ*phoA*, and *recA*	33
ML308-225	Fecal isolate	22
ML308-C2	Mar mutant of ML308-225	This study
KMN14	Derivative of ASS111; contains a chromosomal *marOR*A*::*lacZ* translational fusion derived from mutant *marR2*	This study
KMN18	Derivative of ASS111; contains a chromosomal *marORA*::*lacZ* translational fusion derived from wild-type *marR*	This study
Plasmids		
pSPORT1	Ampicillin resistance; expression vector	Gibco/BRL
pSPOK	Kanamycin resistance derivative of pSPORT1	20
pBluescript	Ampicillin resistance; high-copy-number ColE1-based phagemid	Stratagene
pKMN23	pSPOK bearing a portion of *marRA* in the antisense direction relative to the T7 promoter	This study
pSPOK-*marR*	pSPOK bearing 570 bp of *marOR* from AG100	This study
pSPOK-*marR1*	pSPOK bearing 570 bp of *marOR* from AG102	This study
pDW10	pBluescript-*marA*	This study
pDW11	pBluescript-*marORAB*	This study
pMLB1034	Translational-fusion plasmid	M. Berman and R. Zagursky; reference 8
pKMN14	*marOR*A*::*lacZ* translational fusion in pMLB1034 (*marR2* mutation)	This study
pKMN18	*marORA*::*lacZ* translational fusion in pMLB1034	This study

products were determined by the method of Sanger et al. (32) with the Sequenase sequencing kit (U.S. Biochemicals, Cleveland, Ohio).

Antimicrobial susceptibility. Tetracycline hydrochloride, chloramphenicol, ampicillin, kanamycin, rifampin, nalidixic acid, and norfloxacin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Bacterial susceptibility to antibiotics was assayed by the gradient plate method as described previously (12).

DNA manipulations. Plasmid DNA was prepared with the Wizard Prep Kit (Promega, Madison, Wis.). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) and used under conditions suggested by the supplier. PCR amplification was carried out with a Perkin-Elmer Cetus DNA model 480 thermal cycler. *Taq* polymerase and reagents were provided by Perkin-Elmer Cetus and used as directed.

Transcription of target RNA. Ten micrograms each of the plasmids pDW10 (bearing *marA*) and pDW11 (bearing *marORAB*) was linearized with *Hin*dIII (New England Biolabs) or *Eco*RI (New England Biolabs) in a 50-μl reaction volume containing a 1× dilution of the appropriate buffer and 100 U of restriction enzyme. Cleavage was allowed to proceed at 37°C for 2 h. After this time, the reaction mixture was extracted twice with buffered phenol-$CHCl_3$ (1:1) and twice with $CHCl_3$-isoamyl alcohol (24:1) and precipitated with 3 volumes of a precipitation mixture (66 mM sodium acetate in ethanol). The DNA was collected by centrifugation in a microcentrifuge for 20 min, washed with 70% ethanol, and dried briefly under vacuum. The resultant pellet was resuspended in 20 μl of water and stored frozen until needed.

RNA was transcribed from the linearized plasmids in a 20-μl reaction volume containing 1× supplied buffer; 1 mM (each) rATP, rCTP, and rUTP; 0.9 mM guanosine; 0.1 mM GTP; 2 U of RNasin (Promega) per μl; 2 μg of linearized plasmid; and 2.5 U of T7 RNA polymerase (New England Biolabs) per μl. This reaction mixture was incubated at 37°C for 1 h, and 1 U of RQ1 DNase (Promega) was added. DNA was digested at 37°C for 15 min, and then the RNA was purified with a ProbeQuant G-50 spin column (Pharmacia Biotech) in accordance with the manufacturer's protocol. This procedure produced about 50 μl of solution containing the transcript. At this point, the integrity of the sample of the transcript was checked by electrophoresis in a 1% agarose–Tris-borate-EDTA gel and ethidium bromide staining.

Radiolabelling of target RNA. The transcripts were radiolabelled with ^{32}P in a 20-μl reaction volume containing 10 μl of transcript (one-fifth of transcription product), 2 μl of supplied T4 polynucleotide kinase buffer, 5 μl of [γ-^{32}P]ATP (10 mCi/ml; Amersham), 1 μl of 40-U/μl RNasin, and 2 μl of 10-U/μl T4 polynucleotide kinase (New England Biolabs). After 1 h of incubation at 37°C, the transcript was purified with a spin column as described above, yielding the radiolabelled transcript in a volume of approximately 50 μl. The integrity of the transcript was checked by denaturing polyacrylamide electrophoresis and autoradiography.

Synthesis of oligonucleotides and RNase H mapping of accessible sites in target RNA. Phosphodiester and phosphorothioate oligonucleotides were synthesized on 1- and 10-μmol scales on a Gene Assembler (Pharmacia, Uppsala, Sweden) with standard β-cyanoethylphosphoramidite chemistry, amidites obtained from Cruachem (Glasgow, Scotland), and supports obtained from Millipore (Framingham, Mass.). The random 20-mer phosphodiester contained an equimolar mixture of A, C, G, and T at each position. To assist in identifying portions of the *marORAB* operon that are sensitive to inhibition by antisense oligonucleotides, the technique of RNase H mapping was employed as a probe for the RNA-DNA duplex (17). The reaction was done in a 10-μl reaction volume containing 5 μl of radiolabelled transcript, 1 μl of 10× RNase H buffer (400 mM Tris-HCl [pH 7.4], 40 mM $MgCl_2$, 10 mM dithiothreitol), 0.5 μl of 40-U/ml RNasin, 1 μl of 500 mM random 20-mer (heated and snap cooled), and 1.5 μl of water (17). This mixture was incubated at room temperature for 90 min, and 1 μl of 1-U/μl RNase H (Boehringer Mannheim, Indianapolis, Ind.) was added. Control reactions lacking random 20-mer, RNase H, or both were done in parallel. After 10 min at room temperature, the reaction was quenched by the addition of 10 μl of formamide loading dye. Samples were denatured by heating to 95°C for 5 min, and 7 μl was analyzed by electrophoresis in a 4% polyacrylamide denaturing gel (Fig. 2).

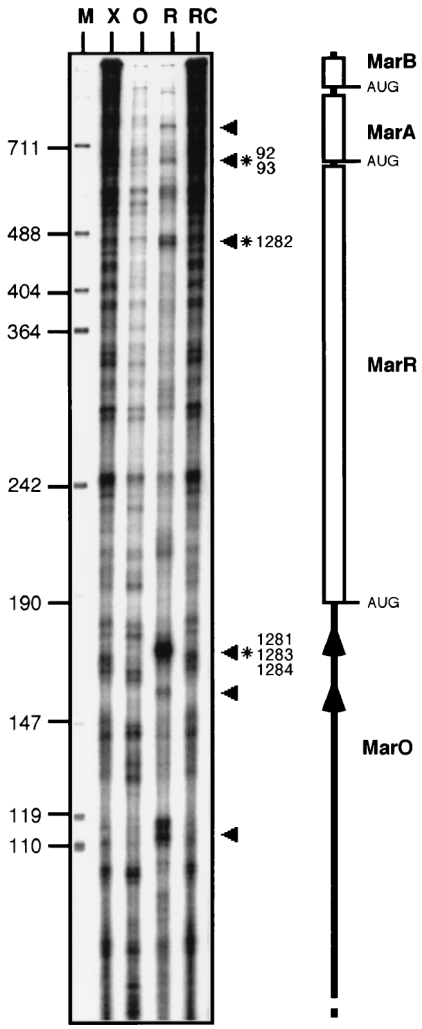

FIG. 2. RNase H mapping of RNA transcribed from the *mar* locus for identification of sites accessible to antisense oligonucleotides. 5′-end-labelled RNA was incubated in buffer (lane X); buffer plus random 20-mer (lane O); buffer, oligonucleotide library, and RNase H (lane R); or buffer plus RNase H (lane RC) as described in Materials and Methods; then it was analyzed by denaturing electrophoresis and visualized by autoradiography. The mobility of the DNA markers is shown on the left (lane M). Those sites appearing only when RNA, oligonucleotide library, and RNase H are present (lane R; see also arrowheads) suggest antisense-oligonucleotide-facilitated RNA cleavage. Antisense phosphorothioate oligonucleotides (identified by numbers in the center) targeted to the sites indicated by asterisks (see Table 4 for exact sequences) were synthesized. The *mar* operon is shown schematically on the far right, aligned with the transcript.

Treatment of *E. coli* with antisense oligonucleotides. Oligonucleotides were introduced into the competent cells (10^5) by a 1-min heat shock (42°C) or through electroporation, and the samples were then incubated at 30°C for 1 or 2 h in LB broth. The transformed cells were used in β-galactosidase assays, colony formation studies, or time-kill experiments.

β-Galactosidase assays. Competent strains KMN14 and KMN18 (50 μl; 10^7 cells) were subjected to heat shock for 1 min with or without the appropriate oligonucleotide and incubated at room temperature for 30 min. LB broth was then added to 1 ml, and the cell-oligonucleotide mixture was then incubated at 30°C for 30 min. For the experiments, KMN18 (wild type) was induced for 30 min with 5 mM sodium salicylate; KMN14 constitutively expressed the *marA*::*lacZ* fusion. Samples of cell-oligonucleotide mixtures were then removed at 1-h intervals and assayed for β-galactosidase by previously described methods (23).

***E. coli* colony formation experiments.** Competent cells of ML308-225-C2 (Mar mutant) (50 μl; 10^7 cells) were subjected to either heat shock at 42°C for 1 min or electroporation. The cell suspension was placed on ice for 2 min and then held at room temperature for 30 min (to allow for association of the antisense oligonucleotide with the mRNA). LB broth (450 μl) was added to the mixture, which was incubated for up to 2 h at 30°C. After 30 min of incubation at 30°C, norfloxacin (at its MIC, 0.5 μg/ml) was added to the cell suspension. At 1 h after exposure to norfloxacin, 100-μl samples of the cell-oligonucleotide mixtures were collected and diluted into phosphate-buffered saline, and the various dilutions were plated onto LB agar. Following overnight incubation at 30°C, the numbers of colonies on the plates were determined. For all experiments, the samples were plated in duplicate or triplicate and all colonies on the plates were counted. Variability in cell counts was ≤5%.

Statistical methods. Significance was determined by performing a one-way analysis of variance. The point of interest decided on at the outset was whether antisense treatment inhibited expression of LacZ in the *marA*::*lacZ* translational-fusion strains relative to its expression in untreated or control oligonucleotide-treated bacteria. Similarly, in norfloxacin cell-killing experiments, the sensitizing effect of antisense oligonucleotides was compared to the results for untreated and control oligonucleotide-treated bacteria. Significance was established for a P value of <0.05.

TABLE 2. Effect of MarR or antisense *marA* on β-galactosidase activities from *marA*::*lacZ* translational-fusion strains

Strain	*marA*::*lacZ* fusion[a]	Cloned gene[b]	β-Galactosidase activity (U)[c]	Fold reduction
KMN14	OR*(A)	None	5,130	
		marR	550	9.3
		marR1	4,278	1.2
		Antisense *marRA*	950	5.4
KMN18	OR(A)	None	2,741	
		marR	59	46.5
		marR1	1,659	1.7
		Antisense *marRA*	56	48.9

[a] *lacZ* translational fusions to *marA* inserted into the chromosome.
[b] All genes were cloned into pSPOK (wild-type *marR* from AG100, mutant *marR* from AG102, and antisense *marRA* on pKMN23). Cells bearing pSPOK plasmids were induced by IPTG (0.5 mM). Values are representative of an experiment done in duplicate.
[c] Method of Miller (23).

RESULTS

Effect of wild-type or mutant *marR* or a portion of the *marRA* gene sequence cloned in the antisense direction on the expression of β-galactosidase activity from *marA*::*lacZ* translational fusions and on antibiotic susceptibility. IPTG induction of wild-type MarR from pSPOK-*marR* reduced the LacZ expression from the two different *marA* translational fusions (mutant *marOR***A*::*lacZ* in KMN14 and wild-type mar*ORA*::*lacZ* in KMN18) (Table 2). Overexpression of mutant MarR had little effect on either fusion (Table 2). The *marRA* antisense product effectively reduced LacZ expression from the fusions, achieving reductions similar to that of wild-type MarR (Table 2).

Wild-type *marR* and pKMN23 (antisense *marRA*) were introduced into the Mar mutant AG102 to determine the effect on antibiotic susceptibility. The antisense construct reduced resistance to multiple antibiotics, although it was less effective than overproduction of MarR (Table 3).

Selection of antisense sequences. RNase H recognizes the DNA-RNA heteroduplex and cleaves RNA. Accessible sites were identified as abundant radiolabelled fragments unique to the complete reaction lane. While there were some background bands generated in the absence of RNase H, novel sites were clearly identified (Fig. 2, lane R). These included both the 5′ untranslated region (bases 1401 to 1450) and the coding region (bases 1708 to 1727) of MarR as well as sites near the translational start site (bases 1890 to 1950) and in the coding region (bases 2040 to 2075) of MarA (numbering as in reference 7). Several antisense sequences complementary to these regions were subsequently synthesized for analysis in *E. coli*. The exact sequences of some of these (identified by number) are given in Table 4. The approximate locations of their binding sites are shown in a graphic representation of the *marRAB* transcript in Fig. 2. The lengths of the RNA fragments produced were calculated by comparison to a radiolabelled DNA restriction ladder. This ladder had been previously calibrated against RNAs of known lengths.

Effect of antisense oligonucleotides on β-galactosidase activity in KMN18 strains induced with 5 mM salicylate. KMN18 was treated with increasing concentrations (4 to 20 μM) of the different antisense oligonucleotides (Table 4) before being incubated with 5 mM salicylate to induce expression of the *lacZ* fusion. Samples were removed after 30- and 60-min periods and assayed for β-galactosidase activity. Two oligonucleotides (92 and 1284) affected *lacZ* expression the most. Little effect was seen with 4 and 10 μM concentrations of these oligonucleotides. However, at 20 μM, oligonucleotides 92 and 1284 significantly reduced LacZ activity in KMN18 (Table 5); these results were reproducible. Oligonucleotides 92 and 1284 also reduced constitutively expressed *marOR**A::*lacZ* activity in KMN14, but to a lesser extent (88 and 79% of control values [data not shown]). Neither of the control oligonucleotides, 101C and 1403 (a scrambled 1284), had a detectable effect.

Selection of a Mar mutant of ML308-225. Antibiotic-resistant mutants of ML308-225 were selected by plating washed overnight cultures onto LB agar containing chloramphenicol (5 μg/ml) and incubating them at 30°C for 48 to 72 h. Single colonies appearing after 48 h were selected and assayed for multiple antibiotic resistance with antibiotic gradient plates. One mutant, ML308-C2, for which the MICs of tetracycline, chloramphenicol, ampicillin, nalidixic acid, and norfloxacin were increased, was chosen for further studies. DNA sequencing confirmed a C→T substitution at amino acid 117 of the sequence encoded by *marR*, which results in a truncated MarR protein. The mutation was designated *marR9*. When wild-type *marR* was cloned into ML308-C2, the MICs decreased to that for the wild-type strain, ML308-225 (data not shown). Northern analysis with a *marA* DNA probe showed overexpression of *marA* in ML308-C2, confirming that it was a Mar mutant (data not shown). These results, in conjunction with the sequence data, support the conclusion that the *marR9* mutation is responsible for the Mar phenotype in strain ML308-C2.

Effects of antisense oligonucleotides on the bactericidal activity of norfloxacin. Competent cells of Mar mutant ML308-C2 were mixed with different amounts of oligonucleotides and 0.5 μg of norfloxacin per ml (equivalent to the MIC), incubated for 1 h at 30°C, and then plated on LB agar and incubated at 30°C overnight. The antisense oligonucleotide 1284 enhanced the killing effect of norfloxacin on ML308-C2 (Table 6). In two separate experiments, there was a greater loss of viability with concentrations of 100 to 200 μM than with lower concentrations (20 to 40 μM). No enhanced norfloxacin killing was seen with control oligonucleotide 1403 at 100 μM, nor did either of the oligonucleotides 1284 and 1403 affect cell viability when tested alone (without norfloxacin) at 100 μM (data not shown). The results demonstrated a reproducible

TABLE 3. Effect of *marR* or antisense *marRA* constructs on antibiotic susceptibility

Strain	Plasmid	MIC (μg/ml)[a]				
		Tetracycline	Chloramphenicol	Ampicillin	Rifampin	Norfloxacin
AG100	None	3.3	4.4	2.6	5.0	0.14
AG102	None	12.5	34.2	17.7	8.8	0.82
AG102	pSPOK-*marR*	3.7	3.3	5.5	1.1	0.32
AG102	pKMN23 (antisense *marRA*)	8.3	16.4	15	2.6	0.64

[a] Determined by the gradient plate method in the presence of IPTG (0.5 mM). MICs are representative of an experiment done in duplicate.

TABLE 4. Oligonucleotides used in this study[a]

Oligonucleotide	Sequence	Target
101C	5′-CTGACTGCCAACTATGAACA-3′	Unrelated control oligonucleotide sequence
92	5′-GCGTCTGGACATCGTCAT-3′	ATG of *marA*
93	5′-ATCGTCATAGCTCTT-3′	Shine-Dalgarno sequence, ATG of *marA*
1281	5′-CTTTTCACATTAGTTGCCC-3′	*marO*
1282	5′-GCGCTTGTCATTCGGGTTC-3′	Coding region of MarR
1283	5′-GTAATTAGTTGCAGAGGATA-3′	*marO*
1284	5′-TAGTTGCAGGAGATAATATTG-3′	*marO*
1403[b]	5′-GTTCAGAGTTAGTAGGAATAT-3′	Scrambled control of 1284

[a] Except that of 101C, all sequences were determined by RNase H analysis in vitro (Fig. 2).
[b] Contains the same nucleotides as 1284, but in a sequence noncomplementary to the RNA.

effect of oligonucleotide 1284, that of increasing the killing effects of norfloxacin on the Mar mutant strain ML308-C2.

DISCUSSION

The emergence of bacteria with resistance to antimicrobial agents has been and continues to be of concern to clinicians, public health officials, and clinical microbiologists. Accordingly, more attention is being paid to new approaches to the resistance problem.

A mutated chromosomal multiple antibiotic resistance locus provides resistance to many structurally unrelated agents, including nalidixic acid, rifampin, penicillins, cephalosporins, and fluoroquinolones (2, 12). The *mar* locus consists of two divergently expressed units, *marC* and *marRAB*, both transcribed from a central operator-promoter site, *marO*. Mar mutants constitutively express the *marRAB* operon due to mutations in *marR* or *marO* (7). In Mar mutants, tetracycline, chloramphenicol, and fluoroquinolone resistances are likely due to a combination of decreased influx through OmpF and other porins and increased or more effective efflux (5, 12, 21). Of note, the *acrAB* operon, which encodes an efflux pump, is upregulated in Mar mutants (26); its deletion leads to loss of much of the drug resistance phenotype (26). The *mar* locus also provides resistance to oxidative stress (3) and organic solvents (4, 15, 36).

A potential approach to antimicrobial therapy is the use of synthetic oligonucleotides which are complementary to and antisense relative to mRNA, leading to inhibition of gene product synthesis. The inhibition generally relies on the ability of the oligonucleotide to bind to a complementary mRNA sequence and prevent translation of the mRNA (9, 24, 35). The mechanism of recognition between two nucleic acid strands is hydrogen bonding of the four nucleotide bases. Synthetic oligonucleotides administered exogenously represent a novel class of therapeutic agents and have been successfully used in both prokaryotic and eukaryotic systems (1, 9, 11, 19, 28–31). For instance, an antisense oligonucleotide complementary to the Shine-Dalgarno ribosomal docking sequence of *E. coli* 16S rRNA inhibited translation of bacterial mRNA in cell extracts derived from *E. coli* (19). Furthermore, experiments using a photoactivatable antisense DNA construct showed suppression of ampicillin resistance in *E. coli* (11). More recently, antisense oligodeoxyribonucleotide phosphorothioates were shown to successfully inhibit growth of a wild-type and a drug-resistant strain of *Mycobacterium smegmatis* (31).

In the present investigation, experiments were conducted to determine if an approach that used antisense oligonucleotides could affect expression of a regulatory protein, namely, MarA (encoded by the *marRAB* operon). Introduction of pKMN23 (an antisense *marRA* plasmid) into both wild-type and mutant *marORA*::*lacZ* fusion constructs effectively repressed LacZ activity. This plasmid construct in the Mar mutant strain AG102 increased susceptibility to multiple antibiotics. Like *marR* itself, the antisense *marRA* increased susceptibility to different

TABLE 5. Effects of antisense oligonucleotides (20 μM) on β-galactosidase activity of salicylate-induced KMN18 bearing *marA*::*lacZ* fusion

Incubation time and oligonucleotide	% of control activity in expt[a]: A (1,667; 1,116[b])	B (1,184; 2,368)	C (4,714; 4,821)	D (3,453; 2,946)	Mean ± SD
30 min					
None	100	100	100	100	100
101C	ND[d]	72	128	78	93 ± 31
92	27	38	95	52	53 ± 30
1284	9	23	50	17	25 ± 18[c]
1403	83	128	ND	ND	105
60 min					
None	100	100	100	100	100
101C	ND	146	80	95	107 ± 35
92	56	117	44	59	69 ± 33
1284	46	45	9	20	30 ± 18[c]
1403	82	144	ND	ND	113

[a] Compared to β-galactosidase activity for salicylate-induced KMN18 without oligonucleotides.
[b] Numbers in parentheses are β-galactosidase activities (in units) of KMN18 treated with 5 mM salicylate without any oligonucleotide for the 30- and 60-min incubations, respectively.
[c] $P < 0.05$ compared to the value for cells treated with 101C or 1403.
[d] ND, not done.

TABLE 6. Effects of increasing antisense oligonucleotide concentrations on bactericidal effect of norfloxacin[a]

Oligonucleotide 1284 concentration (μM)	Norfloxacin (0.5 μg/ml)	% of control colony no. in expt[b]: A	B
0	−	100	100
0	+	71	57
20	+	43	54
40	+	42	58
100	+	27	46
200	+	25	38

[a] ML308-C2 cells were incubated for 1 h at 30°C before being plated onto LB agar.
[b] Control colony numbers on LB agar following a 10^{-5} dilution were averages from duplicate plates and were 691 and 598 for experiments A and B, respectively. Variability in colony counts was ≤5%.

extents depending on the antibiotic tested. Since the target is a regulatory protein, this finding is likely associated with the relative affinity of this regulatory protein for its targets in the *mar* regulon.

Subsequent to these findings, six antisense oligonucleotide phosphorothioates, detected through RNase H studies in vitro and targeted to different mRNA regions of the *mar* locus, along with two control oligonucleotides, were synthesized. The most efficient antisense oligonucleotide overall was 1284, which was complementary to 20 bases upstream of the AUG initiation codon of *marR*, followed by oligonucleotide 92, which was complementary to the AUG start codon of *marA*. Antisense oligonucleotide 1284 enhanced the killing effect of norfloxacin on ML308-C2, in sharp contrast to a lack of effect of the scrambled control 1403.

There was experiment-to-experiment variability (Tables 5 and 6) in the levels of activity of the oligonucleotides; however, both 1284 and 92 were repeatedly shown to be active, unlike the controls. The variability is probably linked to the amounts of oligonucleotides which enter the cell, a focus of current studies.

Although the exact mechanisms whereby antisense oligonucleotides 92 and 1284 exert their inhibitory effects are not known, two possible modes of action have been suggested. The double-stranded oligonucleotide-mRNA hybrid can act through steric hindrance and prevent ribosomes from binding to or scanning the message. Alternatively, inhibition of translation can result from the degradation of the target mRNA by RNase H, an enzyme that hydrolyzes the RNA moiety of RNA-DNA complexes. Because of their nuclease resistance and their ability to elicit RNase H activity, phosphothiorate analogs have been widely used in experiments with antisense oligonucleotides (31, 35).

In summary, antisense oligonucleotides targeted to the *marRAB* operon were shown to be effective in repressing expression of a *marA*::*lacZ* construct, as well as in increasing the killing effect of norfloxacin against a treated Mar mutant strain. Antisense oligonucleotides show potential as novel agents for blocking the expression of specific genes in bacteria.

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