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NorA Plasmid Resistance to Fluoroquinolones: Role of Copy Number and *norA* Frameshift Mutations

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Staphylococcus aureus **NorA protein is a transmembrane multidrug efflux pump that confers low-level resistance to hydrophilic fluoroquinolones. The *norA* gene promoter is active in *Escherichia coli* HB101. We have examined the genetic basis of *norA*-mediated resistance in *E. coli* by introducing a wild-type *norA* gene into HB101 on plasmid pCL1921, pBR322, or pUC18 exhibiting copy numbers that spanned a 22-fold range. Increased ciprofloxacin resistance correlated with *norA* transcript levels seen by Northern (RNA) analysis. Thus, contrary to some reports, a wild-type *norA* gene confers fluoroquinolone resistance in *E. coli* in a copy-number-dependent fashion and does not require mutational activation. Interestingly, a multicopy pUC19*norA* derivative gave transformants exhibiting a range of resistance phenotypes. The *norA* gene of one transformant carried a single base deletion (ATACAAT to AACAAT; the deleted base is underlined) in the putative −10 Pribnow box resulting in a promoter down-regulatory mutation; a second plasmid had acquired a frameshift producing a null mutation at codon 112. These mutations override the dual resistance–growth-inhibitory phenotype of high-copy-number *norA* plasmids. The results have implications for using the standard *E. coli* HB101 system to assess NorA function and potentially for plasmid-borne transmission of *norA*-mediated drug resistance.**

4-Quinolones, and particularly fluoroquinolones such as ciprofloxacin and norfloxacin, are potent antimicrobial agents that act by inhibiting bacterial DNA synthesis (35). Their primary target in *Escherichia coli* is DNA gyrase, the enzyme that catalyzes ATP-dependent DNA supercoiling (3–5). Recent work indicates that DNA topoisomerase IV, responsible for chromosome decatenation, is likely a secondary target in *E. coli* (9, 13). Fluoroquinolones are also active against gram-positive species such as *Staphylococcus aureus* (23). Unfortunately, a major problem associated with quinolone use against *S. aureus* has been the rapid emergence of resistant strains (32). Many staphylococcal strains are already resistant to methicillin, and there is a limited repertoire of effective drugs. This situation has prompted work aimed at understanding the mechanisms of fluoroquinolone resistance.

Three defined genetic loci in *S. aureus* have thus far been implicated in quinolone resistance (11): *gyrA* and *gyrB*, encoding subunits of DNA gyrase (16, 29, 30); *grlA* (possibly allelic with *flqA* [33]), encoding the ParC subunit of topoisomerase IV (2); and *norA*, which specifies a drug efflux pump (12, 20, 21, 34, 37). There is good evidence that chromosomal mutation initially in *grlA* followed by an alteration in *gyrA* can confer high-level quinolone resistance (1). However, the role of *norA* in resistance is less well defined. The *norA* gene was first isolated from a norfloxacin-resistant clinical isolate by screening of a library of chromosomal DNA in plasmid pBR322 for an ability to confer resistance on *E. coli* HB101, a norfloxacin-susceptible strain (11, 34). The gene encodes a 388-residue protein which exhibits 12 putative transmembrane domains (12, 37). NorA belongs to a family of transport proteins that includes the related Bmr multidrug transporter of *Bacillus subtilis* and Tet, the tetracycline resistance pump (15, 19, 20, 34).

Initial studies suggested that *norA* is somehow activated by mutation in drug-resistant isolates. Thus, Oshita et al. attributed resistance of one strain to an Asp-362–to–Ala mutation in NorA (22). Two other resistant strains were shown to carry *norA* promoter mutations that acted to upregulate NorA expression (12, 21). During the course of studies of fluoroquinolone resistance in *S. aureus*, we isolated the *norA* gene from a ciprofloxacin-resistant clinical isolate, *S. aureus* SA47, by screening a plasmid library for drug resistance in *E. coli* HB101 (11, 34). The gene was found to be identical to its counterpart in SA31, the matched parental drug-susceptible strain. This observation led us to examine in detail the molecular basis of *norA*-mediated resistance in *E. coli* and the parameters limiting the utility of this system for studies of *norA* function. We show that *norA* has a dual copy-number-dependent resistance–growth-inhibitory phenotype that has both practical and biological implications.

MATERIALS AND METHODS

Bacterial strains and plasmids. Methicillin-resistant *S. aureus* strains SA31 and SA47 have been described previously (29). *E. coli* HB101 is F$^-$ *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpdL20* (Sm^r) *xyl-5 mtl-1 supE44* lambda$^-$; *E. coli* XL1-Blue is *supE44 hsdR17* (r_K^- m_K^+) *recA1 endA1 gyrA96 thi1 relA1 lac* lambda$^-$ [F′ *proAB lacI*^{q} *lacZ* M15 Tn*10*(Tet^r)]. Plasmids pBR322, pUC18, and pUC19 and phages M13mp18 and M13mp19 were from our laboratory collection; pCL1921 was from David Summers, University of Cambridge, Cambridge, United Kingdom; and pBluescript SK+ was from Stratagene.

Drugs and enzymes. Ciprofloxacin was a generous gift from Bayer, Wuppertal, Germany. Norfloxacin and ampicillin were purchased from Sigma, Ltd. Restriction enzymes, exonuclease III from *E. coli*, T4 DNA ligase, Klenow fragment of *E. coli* DNA polymerase I, *Taq* polymerase, and S1 nuclease from *Aspergillus oryzae* were obtained from New England Biolabs.

Isolation of *norA* genes. Chromosomal DNA from *S. aureus* SA47 was isolated (17), digested with *Hin*dIII, ligated into *Hin*dIII-digested pBR322, and used to

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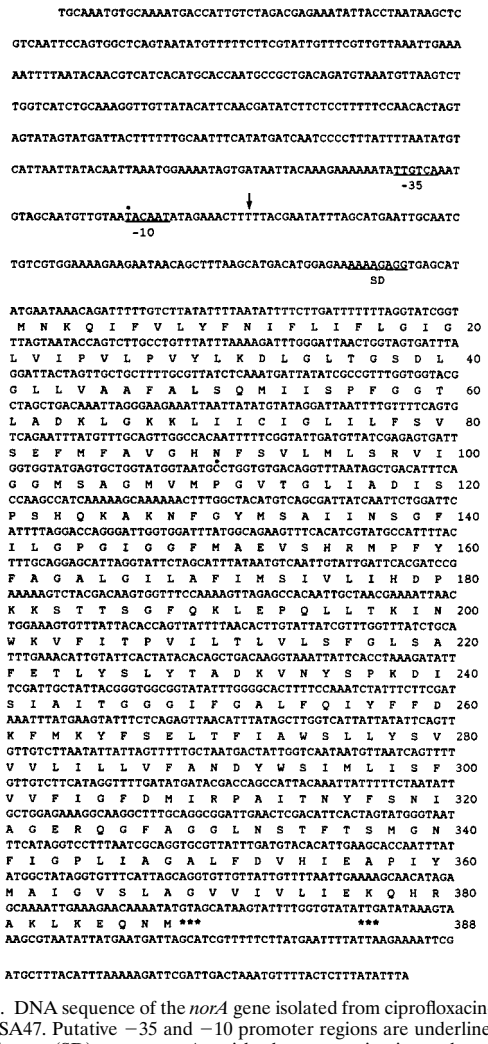

FIG. 1. DNA sequence of the *norA* gene isolated from ciprofloxacin-resistant *S. aureus* SA47. Putative −35 and −10 promoter regions are underlined, as is a Shine-Dalgarno (SD) consensus. Asterisks denote termination codons. The vertical arrow denotes a T in the promoter region that is altered to A or to G in two *norA* promoter mutants (10, 21). Closed circles identify nucleotides deleted in the down-regulated *norA* genes carried by plasmids pSL1000 and pSL1001 (see the text). The deduced amino acid sequence of NorA47 is given below the nucleotide sequence. Numbers on the right indicate amino acid residues.

transform *E. coli* HB101 (7). Bacteria were selected on Luria-Bertani (LB) plates containing 100 μg of ampicillin per ml and 0.1 μg of ciprofloxacin per ml. Plasmid pSS47 from one drug-resistant colony was purified (25) and characterized by restriction enzyme digestion. Plasmid pSS31, carrying the *S. aureus* SA31 *norA* gene, was obtained by colony hybridization (25) of a size-selected (4.8 to 5.5 kb) pBR322 library of SA31 *Hin*dIII fragments in HB101. The probe used was the pSS47 *norA* insert radiolabeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham International) by random primer extension with the Multiprime DNA Labelling kit (Amersham International). Hybridization of Hybond N filters (Amersham) was carried out overnight at 60°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1.5% dried milk. High-stringency washes were performed at 60°C in 1× SSC–0.1% sodium dodecyl sulfate (SDS). For *norA* PCR, 5′AGTGATGAATTCAAAGAAAAAATA (nucleotide positions −153 to −130 upstream of the start ATG, with an artificial *Eco*RI site at position −147) and 5′ATCAATCATAATATTAAGCTTTAC (complementary to positions +1198 to +1221 with an artificial *Hin*dIII site at position +1206) were used as primers. PCRs were carried out with *S. aureus* or *Staphylococcus epidermidis* chromosomal DNA as the template as previously described (29, 30) with the following modifications: 50 ng of template DNA was used and the annealing temperature was 50°C. Products were confirmed as *norA* by Southern blotting (28).

NorA plasmids. Relative plasmid copy numbers in *E. coli* HB101 were determined by a rapid miniprep method (25). Plasmid DNA was isolated from bacterial cultures in LB broth (3 ml) containing ampicillin (100 μg/ml) grown with aeration at 37°C to an optical density at 600 nm of 0.9. After *Hin*dIII digestion and electrophoresis in 0.8% agarose, DNA bands were stained with ethidium bromide and photographed under UV illumination and relative intensities of *norA* fragments were determined by laser densitometry using the Macintosh Deskscan and Collage software packages. Plasmids pSL1000 and pSL1001 were obtained as follows. A 1.8-kb *Mae*II fragment bearing the *norA47* gene was isolated from plasmid pSS47 and cloned into the *Cla*I site of plasmid pBR322. The resulting plasmid, pSL500, was digested at *Eco*RI and *Sph*I sites flanking the *norA* gene, and the released insert was ligated into *Eco*RI-*Sph*I-cut pUC19^{+}, i.e., pUC19 carrying three tandem copies of the sequence 5′GCTTAATTAATTA AGC (specifying a stop codon in all three reading frames) cloned (downstream of the *Sph*I site) into the *Hin*dIII site that had been filled in using Klenow fragment and the four dNTPs. Transformation of *E. coli* HB101 produced many ampicillin-resistant colonies, two of which yielded plasmids pSL1000 and pSL1001.

Northern (RNA) blotting. Total cellular RNA was isolated from *S. aureus* essentially by the method of Gustafson et al. (6). Its concentration was determined from the A_{260}, and its integrity was checked by electrophoresis in a 1.0% agarose gel. For Northern blots, total RNA (10 μg per lane) was denatured in a solution containing glyoxal and dimethyl sulfoxide and separated by electrophoresis in a 1.2% agarose gel (18). RNA was transferred to a Hybond N nylon membrane and hybridized with an 881-bp *Rsa*I *norA* probe (nucleotide positions +177 to +1058) that had been labeled with [α-^{32}P]dCTP by random priming. Hybridization was carried out at 42°C overnight in a solution containing 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, and 100 μg of denatured salmon sperm DNA per ml. High-stringency washes were carried out as described above for Southern blotting prior to radioautography over several days at −70°C with intensifying screens.

DNA sequence analysis. Nucleotide sequences for both strands of the *norA* genes in plasmids pSS47 and pSS31 were determined by the dideoxy chain termination method (26) using the Sequenase version 2.0 DNA kit (U.S. Biochemicals) and [α-^{35}S]dATP (Amersham) on M13 and plasmid Bluescript SK+ subclones, respectively. For sequencing of pSL1000 or pSL1001, an exonuclease III-S1 method was used on *Hin*dIII-*Sph*I-cut plasmids to generate unidirectional nested 3′ truncations of the *norA* gene as described in reference 23a. Only the recessed 3′ end of the unique *Hin*dIII site downstream of *norA* is digested by exonuclease III: the protruding 3′ terminus of the *Sph*I site (3′ of the *Hin*dIII site) is not attacked, thus protecting vector sequences. Briefly, *Hin*dIII-*Sph*I-cut DNA (5 μg) was digested with *E. coli* exonuclease III (200 U) at 17°C (reaction volume, 60 μl). Samples (2.5 μl) were removed at intervals of 30 s (for small deletions) or 2 min (for sequencing purposes) and added on ice to tubes containing S1 nuclease (4 U) in its reaction buffer (7.5 μl). Treatment with S1 nuclease and Klenow fragment of DNA polymerase I, plasmid self-ligation, and transformation of *E. coli* HB101 were all as described in the Promega protocol. Plasmids with truncated *norA* genes were isolated and subjected to double-stranded DNA sequencing using the pUC19 reverse primer, 5′CAGGAAA CAGCTATGAC, or primers specific to internal *norA* sequences.

Bacterial growth inhibition. Ciprofloxacin MICs were determined by incubation on LB plates containing 100 μg of ampicillin per ml for 20 h at 37°C. For *norA*-associated growth inhibition studies, equal inocula of HB101 plasmid transformants were added to LB broth (20 ml) containing 100 μg of ampicillin per ml and incubated at 37°C with aeration. Bacterial growth was measured by optical density at 600 nm.

RESULTS

Cloning *norA* genes from *S. aureus* SA47 and SA31. We used the standard method of screening a pBR322 library of genomic *Hin*dIII fragments in *E. coli* HB101 to obtain the *norA* gene from *S. aureus* SA47, a highly ciprofloxacin-resistant clinical isolate with a ciprofloxacin MIC of 16 μg/ml (29). Plasmid pSS47, carrying a 5.2-kb *Hin*dIII *norA* insert, was isolated and when retransformed into HB101 was sufficient to confer ciprofloxacin resistance. DNA sequence analysis of an internal 2.7-kb *Sph*I-*Kpn*I fragment confirmed the presence of the *norA* gene (*norA47*) (Fig. 1). We could not be sure from the literature that the same approach would be successful for SA31, the matched ciprofloxacin-susceptible (MIC, 1 μg/ml) pretreatment parent to SA47. Therefore, plasmid pSS31 bearing the *norA31* gene was isolated from a size-selected library of 4.8- to 5.5-kb *Hin*dIII fragments in pBR322 by colony hybridization with a radiolabeled *norA* probe.

Drug resistance and bacterial growth inhibition are copy-number-dependent *norA* phenotypes. To examine the roles of *norA47* and *norA31* in *norA*-mediated resistance, we compared the phenotypes of 5.2-kb *norA Hin*dIII fragments cloned into

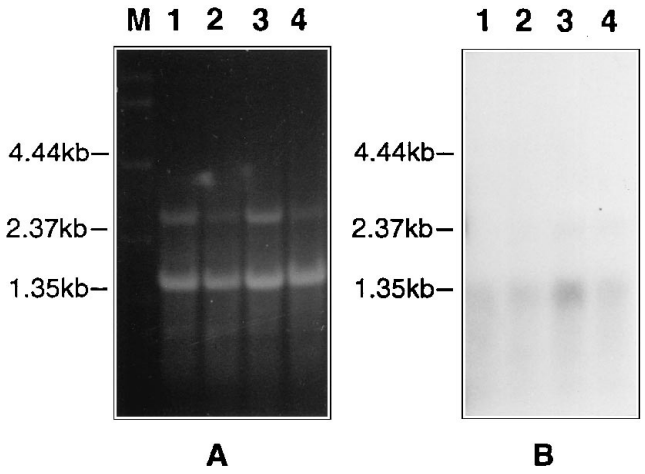

FIG. 2. Northern analysis of *norA* expression in *S. aureus* strains SA31 and SA47. Total RNA from matched isolates SA31 and SA47 (A) was hybridized with a radiolabeled *norA* probe (B). Lanes 1 and 2, RNA from exponentially growing SA31 and SA47; lanes 3 and 4, RNA from SA47 cultures exposed for 40 min to 0.25 and 0.5 μg of ciprofloxacin per ml before harvesting, respectively. Positions of RNA size markers run in lane M are indicated on the left of each panel.

the single *Hin*dIII site of ampicillin resistance plasmids pCL1921 (4,600 bp), pBR322 (4,361 bp), and pUC18 (2,686 bp), which have reported copy numbers of approximately 5, 15 to 20, and 200, respectively (14). These constructs were transformed into *E. coli* HB101, and their relative copy numbers were determined by quantitative agarose gel electrophoresis (see Materials and Methods). Consistent with published work for other *E. coli* strains, five independent experiments gave relative copy numbers for pCL1921, pBR322, and pUC18 of 1 to 2.2 to 22, ratios not significantly altered by the presence of *norA* inserts. The ciprofloxacin MICs for strains containing pCL1921, pBR322, and pUC18 vector DNA were in each case 0.005 μg/ml. Insertion of the *norA* gene from SA47 increased the MIC from 0.005 to 0.15 (pCL1921*norA*), 0.20 (pBR322*norA*, i.e., pSS47), and 0.55 μg/ml (pUC18*norA*). We were surprised that the same copy-number-dependent increases in resistance were seen with the *norA31* insert, and this observation led us to sequence the *norA31* structural gene and its promoter. The region was identical to that of *norA47*, suggesting that the wild-type *norA* gene is sufficient to confer resistance in *E. coli*. Furthermore, levels of expression of the 1.35-kb *norA* transcript in *S. aureus* SA31 and SA47 were similar (Fig. 2). Exposure of SA47 to 0.25 μg of ciprofloxacin per ml resulted in a small induction of *norA* transcripts. At this level of drug, growth of SA47 was not markedly impaired, and the strain's Ser-84–to–Leu GyrA resistance mutation appears to rule out secondary effects via gyrase inhibition. Although further work will be necessary to examine the role of inducible *norA* expression, changes in the primary structure of the *norA* gene itself do not appear to be a factor in resistance of strain SA47.

Interestingly, we noticed that high-copy-number *norA* plasmids also displayed a novel growth-inhibitory phenotype (Fig. 3). Growth was significantly impaired by the presence of the *norA31* insert in pBR322 and especially in pUC18. The relevance of the linked resistance–growth-inhibitory phenotypes of *norA* genes in *E. coli* is considered later.

Instability of pUC19*norA* constructs in *E. coli*. During attempts to overexpress *norA* protein in *E. coli* for biochemical studies by exploiting constitutive expression from a high-copy-number plasmid, we noted that a pUC19*norA* construct, when assembled and used to transform HB101 to ampicillin resistance, generated transformants with the expected plasmid restriction map but exhibiting very different ciprofloxacin MICs. Of two clones selected for study, one bearing plasmid pSL1000 was inhibited by ciprofloxacin at 0.25 μg/ml, whereas the other, containing plasmid pSL1001, was entirely susceptible, with a ciprofloxacin MIC of 0.005 μg/ml. The differing ciprofloxacin MICs of these transformants were unexpected: we had anticipated uniform MICs of 0.55 μg/ml as seen for the closely related plasmid pUC18*norA* (see the previous section).

Northern blot analysis was used to compare transcript levels in *E. coli* transformants containing pSL1000 and pSL1001 with those in transformants containing other plasmids bearing *norA* inserts (Fig. 4). The 1.35-kb *norA* transcript was present in all *norA* transformants but was absent from HB101 containing pUC18. Transcript levels for pCL1921, pBR322, and pUC18 carrying the 5.2-kb *Hin*dIII *norA* increased with copy number (lanes 2 to 6). Plasmid pSL1000 produced resistance and transcript levels similar to those produced by pSS47 (lanes 5 and 9). However, paradoxically, although HB101 containing pSL1001 was fully sensitive to quinolones, the cells displayed hyperexpression of *norA* (lanes 10). In a manner similar to that of previous studies of *norA* expression in *S. aureus* and *E. coli* (12, 21), there was evidence of transcript processing and degradation. For our *E. coli* experiments, this is unlikely to have arisen from RNA degradation during isolation: intact 16S and 23S rRNA bands were present in the RNA samples used (Fig. 4a).

Frameshift mutations in the pUC19*norA* gene and its promoter. The sequence of the pSL1000 *norA* gene and its promoter was identical to that in pSS47 except for one nucleotide change: a deletion of the T (underlined) in the putative Pribnow box sequence at −10 altering the sequence from ATA CAAT to AACAAT (Fig. 1). There were no changes in the coding region. DNA sequence analysis of plasmids pSS31, pCL1921*norA*, pUC18*norA*, and pSL500 showed that they all retained the wild-type promoter region (data not shown). The SL1001 *norA* sequence was identical to that of pSS47 except for a deletion of a C nucleotide at position 328 in the coding sequence (Fig. 1). This frameshift mutation (the deleted nucleotide is underlined) converted the reading frame from ATG CCT GGT GTG ACA (Met-109 Pro Gly Val Thr) to ATG CTG GTG TGA (Met-109 Leu Val Stop). pSL1001 *norA* had the wild-type promoter sequence. To determine if the pSL1001

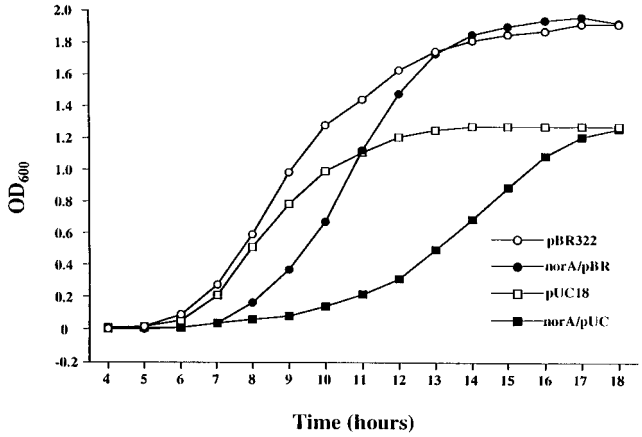

FIG. 3. High-copy-number *norA* plasmids inhibit growth of *E. coli* HB101. The *norA* gene isolated from *S. aureus* SA31 was used. OD_{600}, optical density at 600 nm.

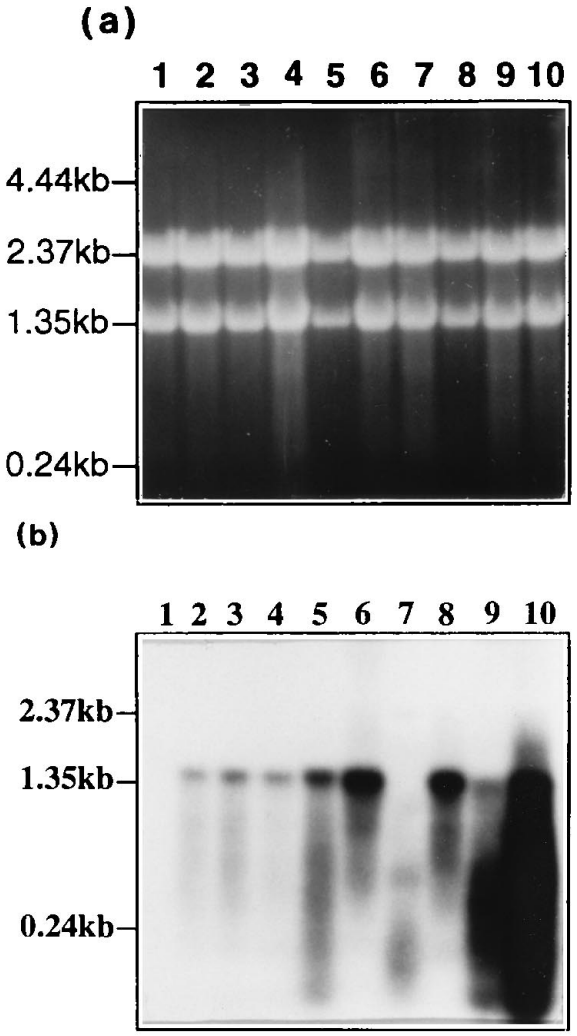

FIG. 4. *norA* expression in *E. coli* HB101. Total RNA, purified from *E. coli* HB101 transformed with various *norA* plasmids, was separated on a denaturing formamide gel (a) and hybridized with a *norA* probe (b). The lanes contain RNA from HB101 (lane 1) or HB101 transformed with pCL1921*nor*A31, pCL1921*nor*A47, pBR322*nor*A31, pBR322*nor*A47, pUC18*nor*A31, pUC18 (control), pSL500, pSL1000, or pSL1001 (lanes 2 to 10, respectively). The positions of RNA size markers are shown on the left.

frameshift generates a null mutation, a series of pSL1000 derivatives bearing progressive 3′ truncations of the *norA* gene were obtained by exonuclease III-nuclease S1 digestion (see Materials and Methods). Removal of up to 13 residues of the highly charged C terminus of NorA could be tolerated with full retention of drug resistance: truncations beyond Ile-375 into the putative 12th transmembrane helix resulted in loss of function, implying that the termination codon at position 112 of pSL1001 *norA* produces a truncated inactive NorA protein. Thus, pUC19*norA* plasmids acquire frameshift mutations generating a range of resistance phenotypes.

DISCUSSION

Plasmid studies with *E. coli* have played a pivotal role in both the discovery and the functional analysis of the *S. aureus norA* gene (10, 12, 21, 34, 37). We show in this paper that plasmid-borne wild-type *norA* genes confer ciprofloxacin resistance in *E. coli* in a manner critically dependent on plasmid copy number. The resistance mechanism in *E. coli* involves enhanced transcription and does not require mutational activation of the *norA* gene or its promoter. The activity of the wild-type *norA* gene in *E. coli* has also been observed recently by Ng et al. (21). Thus, the ability to clone *norA* in *E. coli* is not informative about the likely contribution of *norA* to resistance in *S. aureus*. This result is important because *norA* is normally present in many (and perhaps all) strains of *S. aureus*; has a homolog in other staphylococcal species, including *S. epidermidis* (28a, 36); and thus will be encountered frequently during screening for resistance in *E. coli*. Although there were no differences in the primary sequence of *norA* in SA47 and SA31, our work with *E. coli* could not rule out the possibility of *norA* upregulation in *S. aureus* SA47. Two examples of constitutive upregulation due to *norA* promoter mutations are already known (Fig. 1), and recent elegant work (10) has described a mutation in *S. aureus* unlinked to *norA* that confers quinolone-inducible *norA* expression. Northern analysis of SA31 and SA47 showed no differences in basal *norA* expression, but ciprofloxacin induced a severalfold increase in expression in SA47 that could contribute to resistance (Fig. 2). Further work will be needed to examine these aspects of *norA* function in *S. aureus.*

Aside from its use in direct selection for *norA* genes from clinical isolates, the *E. coli* system offers the attractive possibility of expressing NorA protein at high levels for biochemical work. So far there have been no reports of successful expression and purification of NorA protein. During the construction of a pUC19*norA* derivative for expression-deletion analysis, we observed that its propagation in *E. coli* led to the spontaneous acquisition of frameshift mutations in the *norA* gene generating transformants with a range of resistance phenotypes. One pUC19*norA* plasmid, pSL1000, conferring intermediate ciprofloxacin resistance and *norA* transcript levels, had a wild-type *norA* gene that had undergone deletion of the first T in the TACAAT Pribnow box of the promoter, generating the sequence AACAAT. The T at the −10 position is highly conserved among bacterial promoters: its mutation to A has been examined in other systems and is known to produce defective transcription initiation (24, 38). The mutation did down-regulate transcription: *norA* transcript levels for pSL1000 were much lower than those arising from a second pUC19*norA* isolate, pSL1001 (Fig. 4). The latter plasmid had the wild-type *norA* promoter but had acquired a *norA* frameshift producing a termination signal (and null mutation) immediately after codon 111 (Fig. 1). Production of a truncated nonfunctional NorA protein would explain the quinolone sensitivity of HB101 (pSL1001) despite very high *norA* transcript levels (Fig. 4). It appears that high-copy-number *norA* plasmids are unstable in *E. coli*, leading to the selection of spontaneous frameshift mutations in *norA*. This phenotype has not been previously reported, and it imposes limits on the utility of the widely used *E. coli* HB101 system for identification, overexpression, and functional dissection of candidate *norA* resistance genes from *S. aureus.*

Our data indicate that modest upregulation of *norA* expression through promoter mutations or through plasmid copy number can be tolerated in *E. coli* whereas high-level expression from pUC19-based plasmids is selected against. It is plausible that this selection involves escape from the growth inhibition that we observed for high-copy-number *norA* plasmids (Fig. 3). Whatever its origin, the potential genetic instability of *norA* plasmids must be borne in mind when *norA* is manipulated for expression work with *E. coli*. Plasmid pSL1000 produces *norA* transcript levels similar to those of pSS47 (Fig. 4), is stable in *E. coli*, and should facilitate deletion-expression studies. We have used it to show that in a manner similar to

that of the tetracycline resistance pump (27), an intact 12th transmembrane sequence in NorA is necessary for activity.

Finally, *norA-E. coli* is one of the few systems in which fluoroquinolone resistance can be plasmid mediated and exert a dominant phenotype. By contrast, resistance due to *gyrA* requires a mutation and is recessive (8). In principle, plasmid capture of a wild-type *norA* gene could allow horizontal transmission of quinolone resistance in nature, which would be a worrying development. Surprisingly, despite intensive clinical use of fluoroquinolones over a substantial period, there has been only one report (thus far unsubstantiated) of a resistant isolate bearing a *norA* plasmid (31; reviewed in reference 23). This may simply reflect the poorly studied molecular epidemiology of *norA*-promoted resistance. Alternatively, multicopy *norA* plasmids by inhibiting bacterial growth may be disadvantageous in vivo and rarely selected.

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