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Cloning and Characterization of the Catalase Gene of *Neisseria gonorrhoeae*: Use of the Gonococcus as a Host Organism for Recombinant DNA

STEVEN R. JOHNSON,* BRET M. STEINER, AND GOLDIE H. PERKINS

Division of AIDS, STD, and TB Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

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The structural gene for the catalase of *Neisseria gonorrhoeae* was cloned into a Kat$^-$ strain of that organism by using a recombinant vector derived from one of the β-lactamase-specifying plasmids found in that organism. The *kat* gene was then successfully subcloned into both pUC8 and pGB2, transformed into *Escherichia coli*, and shown to complement the *E. coli katE* mutants UM2 and UMR1. The gene was subsequently mutagenized and returned to the gonococcus to generate a Kat$^-$ strain that was phenotypically identical to the strain originally used to clone the gene. The sequence of the gene and the derived amino acid sequence showed that the gonococcal *kat* gene closely resembles the *hktE* gene of *Haemophilus influenzae*. The sequence of the promoter region of the gonococcal *kat* gene is unusual and may explain the extremely high, loosely regulated expression of the gene.

Neisseria gonorrhoeae is the causative agent of gonococcal urethritis, one of the most common sexually transmitted diseases. *N. gonorrhoeae* colonizes and invades the mucous membranes of the human genital tract, causing a considerable influx of phagocytic cells into the colonized area. Such professional phagocytes can destroy bacteria by using an NADPH oxidase to produce superoxide, which reacts to produce hydrogen peroxide and hydroxyl radicals (9, 15, 17, 39). Despite the intense host reaction, gonococci can be cultivated from urethral exudates containing large numbers of phagocytic cells, and experimental data indicate that gonococci can survive after being phagocytized (7, 29). Among the neisseriae, *N. gonorrhoeae* is unusual in that the vast majority of isolates identified as *N. gonorrhoeae* produce large amounts of catalase and expression of the enzyme appears weakly responsive to induction, with the level of activity increasing twofold after exposure of the gonococci to hydrogen peroxide (1, 41). It has been hypothesized that the large amounts of catalase produced by *N. gonorrhoeae* effectively convert hydrogen peroxide into water and molecular oxygen, affording the organism direct protection against damage by hydrogen peroxide and indirect protection against damage by hydroxyl radicals formed from hydrogen peroxide produced by phagocytes (15). In contrast to experimental results obtained with catalase-deficient mutants of *Bordetella pertussis* and *Haemophilus influenzae*, evidence from in vitro studies suggests that catalase affords *N. gonorrhoeae* a degree of protection after phagocytosis (4, 11, 41). The ability to produce high levels of catalase also improves the survival of *N. gonorrhoeae* in the presence of H_2O_2-producing strains of *Lactobacillus acidophilus*, which suggests that catalase may contribute significantly to the ability of *N. gonorrhoeae* to colonize tissues of the female genital tract (40).

Previously, we described a catalase-deficient mutant of *N. gonorrhoeae* isolated from a culture taken from an infected patient. We were able to demonstrate that this mutant was more sensitive to hydrogen peroxide in vivo than were normal Kat$^+$ *N. gonorrhoeae* strains (19). While the data strongly suggested that the catalase deficiency resulted from mutation or loss of the *kat* structural gene, we were not able to confirm this, and we were not able to confirm that the extreme sensitivity of the Kat$^-$ isolate to hydrogen peroxide resulted solely from the absence of catalase. Since *N. gonorrhoeae* lacks the sophisticated and flexible systems available for genetic analysis of other organisms, it is often necessary to clone genes of the gonococcus to establish their role in the biology of the organism. The added benefits of cloning the gonococcal catalase gene include the opportunity to determine the sequence of the gene and the sequences that control its expression and to compare such sequences with those of other catalase genes, perhaps explaining some of the unusual properties of gonococcal catalase and its expression (1, 41). In addition, we are interested in determining the nature of the mutation present in the catalase-deficient isolate described previously, and cloning the wild-type gene is a prerequisite to isolating and analyzing the mutant allele.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains and plasmids used, their pertinent genetic markers, and derivations are given in Table 1. All strains were maintained in Trypticase soy broth with 20% glycerol at −70°C. All strains of *N. gonorrhoeae* were grown under 5% CO_2 on GC base agar (GCBA) containing 1% IsoVitaleX (Baltimore Biological Laboratories, Cockeysville, Md.) and 0.5% fetal bovine serum. All strains of *Escherichia coli* were grown in brain heart infusion broth (Difco, Detroit, Mich.) or on meat extract agar (28).

Nucleic acid purification and analysis. Total DNA from all bacterial strains was prepared as described previously (14). Large amounts of plasmid DNA were purified from cleared lysates by dye-buoyant density centrifugation (10). The plasmid content of bacterial strains was determined by subjecting small amounts of crude plasmid DNA prepared from cleared lysates to agarose gel electrophoresis (25).

Cloning of *kat* and recombinant DNA techniques. All enzymatic manipulations of DNA for analytic purposes and the construction of recombinant plasmids were performed as described by Maniatis et al. (24). *E. coli* strains to be used as recipients were made competent as described elsewhere (18), and piliated, competent gonococcal cultures were selected on the basis of colony morphology (37). Plasmid pCa2 (Fig. 1), constructed by fusing in vitro the previously described 7.2-kb β-lactamase plasmid (33, 37) and the gonococcal cryptic plasmid (20) at *Hin*fI sites, was used as the vector. pCa2 was able to transform *N. gonorrhoeae* 2374 to penicillin resistance at a frequency 10^4-fold greater than that for the

* Corresponding author. Mailing address: Centers for Disease Control and Prevention, DASTLR, MS D13, Atlanta, GA 30333.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant marker or genotype	Comments
N. gonorrhoeae		
2821	Str^r Rif^r Nal^r	From 28 B1 (18)
23744	Rif^r Kat$^-$	From 2374 (18)
23752	Spc^r	Spc^r transformant with pCaK12-2 DNA
23753	Spc^r Kat$^-$	Spc^r transformant with pCaK12-1 DNA
E. coli		
DH5aMCR	*lacZM15 hsdR hsdM mcrA mcrB mcrC mrrA*	
UM2	*lacY1 leuB6 proC83 xthA rpsL109 katE2 katG15*	21, 23
UMR1	As above but RecA$^-$ Tet^r	From NK5304 × UM2
UM56-64	*lacY1 leuB6 proC83 xthA rpsL109 katF3 katG16*	22
Plasmids		
pCa2	Pen^r	This paper
pUC8	Pen$^+$	
pGB2	Spc^r/Str^r	8
pHP45Omega	Pen^r Spc^r/Str^r (Omega fragment)	32
pCaK6	Pen^r Kat$^+$ (cloned *kat* gene)	This paper
pCaK12	Pen^r Kat$^+$	This paper
pCaK12-1	Pen^r Spc^r Kat$^-$	This paper
pCaK12-2	Pen^r Spc^r Kat$^-$	This paper
pUKP21	Pen^r Kat$^+$	This paper
pUKP21-1	Pen^r Kat$^+$ (30-fold reduction)	This paper
pUKP21-2	Pen^r Kat$^+$ (40-fold reduction)	This paper
pUKP27	Pen^r	This paper
pUKP31	Pen^r	This paper
pGB2Kat1	Spc^r Kat$^+$	This paper

β-lactamase plasmid alone (37); this presumably occurs as the result of specific DNA uptake sequences present in the cryptic plasmid sequences (5, 14).

DNA from strain 2821, partially digested with *Rsa*I, was ligated into *Pvu*II-cleaved pCa2 DNA. The ligation reaction was used to transform the Kat$^-$ strain 23744 (19) to penicillin resistance (1.2 μg/ml). Kat$^+$ colonies were detected by dripping 3% H_2O_2 onto individual colonies with a hypodermic syringe. Colonies producing catalase were picked and streaked onto GCBA supplemented with IsoVitaleX, serum, and penicillin. The Pen^r Kat$^+$ isolates were screened to determine if they contained plasmids larger than pCa2 (11.4 kb). Such plasmids were considered to contain inserts of gonococcal chromosomal DNA that potentially included the *kat* gene. Crude plasmid DNA was also used to transform strain DH5aMCR to resistance to 200 μg of penicillin per ml. The Pen^r DH5a colonies were flooded with 3% H_2O_2 and observed for overproduction of catalase. Colonies scored as high-level catalase producers were those that bubbled immediately upon exposure to 3% H_2O_2. This phenomenon was more easily detected on GCBA supplemented with IsoVitaleX, serum, and 200 μg of penicillin per ml, because DH5aMCR transformed with pCa2 appeared to exhibit little catalase activity when grown on GCBA.

Analysis of recombinant plasmids and localization of the gonococcal *kat* gene. The *kat* gene was localized within the initial inserts obtained in pCa2 by digesting recombinant plasmid DNA with restriction enzymes to detect overlapping sequences. Homology among restriction fragments from inserts was determined by hybridizing Southern transfers (36) of agarose gels with the appropriate ^{32}P-labeled (12), purified restriction fragment. Similarly, gonococcal chromosomal sequences homologous with the cloned DNA fragments were detected by hybridizing ^{32}P-labeled fragments of the cloned DNA with chromosomal DNA digested with restriction endonucleases subjected to agarose gel electrophoresis and transferred to nylon membranes (Schleicher & Schuell, Keene, N.H.) (36). All hybridizations were conducted under stringent conditions as described previously (18). The Omega fragment of pHP45 Omega, which confers Spc^r (32), was inserted into cloned DNA to determine more precisely the location of *kat*. Plasmid DNA was partially digested with *Eco*RI, and the full-length linear plasmid was separated from other digest products. The Omega fragment with *Eco*RI ends was ligated with the plasmid. Catalase production by Spc^r transformants was determined as above.

Sequencing of cloned gonococcal DNA. *Eco*RI fragments from pUKP21, which contained the gonococcal catalase gene subcloned from pCaK12, were further subcloned into pUC8 to generate pUKP27 and pUKP31. The inserts contained in the three plasmids were sequenced by the chain termination technique of Sanger et al. (35) with double-stranded plasmid DNA. Successive primers were used to walk the inserts in all three plasmids.

Determination of enzymatic activity. The amounts of catalase activity present in extracts of sonically disrupted, log-phase bacteria were determined by the method of Beers and Sizer (2). Catalase activity present in extracts was also determined by staining nondenaturing polyacrylamide gels for enzymatic activity after electrophoresis as described elsewhere (26). The protein content of samples was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.) with a bovine serum albumin standard (Bio-Rad, Richmond, Calif.).

Determination of the in vitro sensitivity of gonococci to hydrogen peroxide. The biological consequences of the absence of catalase in constructed mutants of *N. gonorrhoeae* were determined by testing the sensitivity of these mutants to hydrogen peroxide as described elsewhere (16, 19).

RESULTS

Cloning of gonococcal catalase in *N. gonorrhoeae*. We initially attempted to clone the *kat* gene of *N. gonorrhoeae* by conventional methods with the vector plasmid pUC8 and the host strain *E. coli* DH5aMCR. More than 60,000 colonies containing putative recombinant plasmids were screened for the gonococcal catalase gene without success.

Since we earlier demonstrated that the Kat mutation present in 85-015, a Kat$^-$ isolate of *N. gonorrhoeae*, could be transferred to any desired gonococcal strain by congression and that the resulting Kat$^-$ strain could be subsequently transformed to Kat$^+$, we attempted to directly clone the *kat* gene from strain 2821 to strain 23744, a strain specifically constructed for that purpose, by using the chimeric vector plasmid pCa2 as described in Materials and Methods. A total of 5,800 Pen^r colonies of strain 23744 were screened for catalase production, and 12 Kat$^+$ colonies that contained plasmids that exceeded 11.4 kb were obtained. Five Per^r Kat$^+$ colonies contained plasmids indistinguishable from pCa2. Two plasmids designated pCaK6 and pCaK12 were selected for further study. pCaK6 and pCaK12 plasmid DNA purified from strain 23744 gave Pen^r transformants with 23744 as the recipient at a frequency similar to that observed for pCa2 (0.001 transformant per CFU),

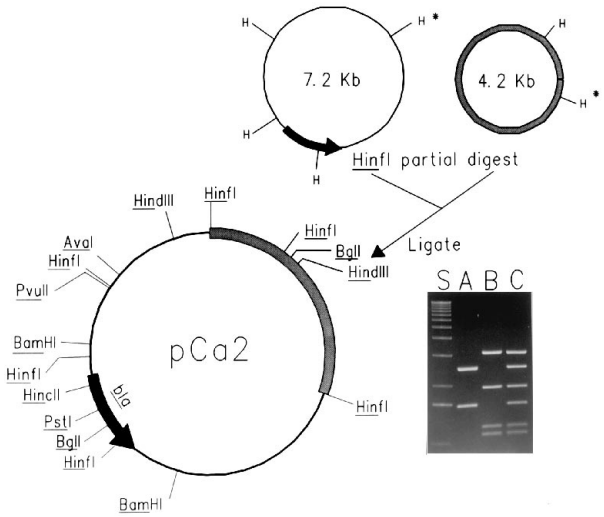

FIG. 1. Construction and restriction map of plasmid pCa2. The inset shows the *Hin*fI digestion patterns obtained from the 4.2-kb cryptic plasmid (lane A), the 7.2-kb β-lactamase plasmid (lane B), and the vector plasmid pCa2 (lane C). Lane S contains the standard kilobase ladder.

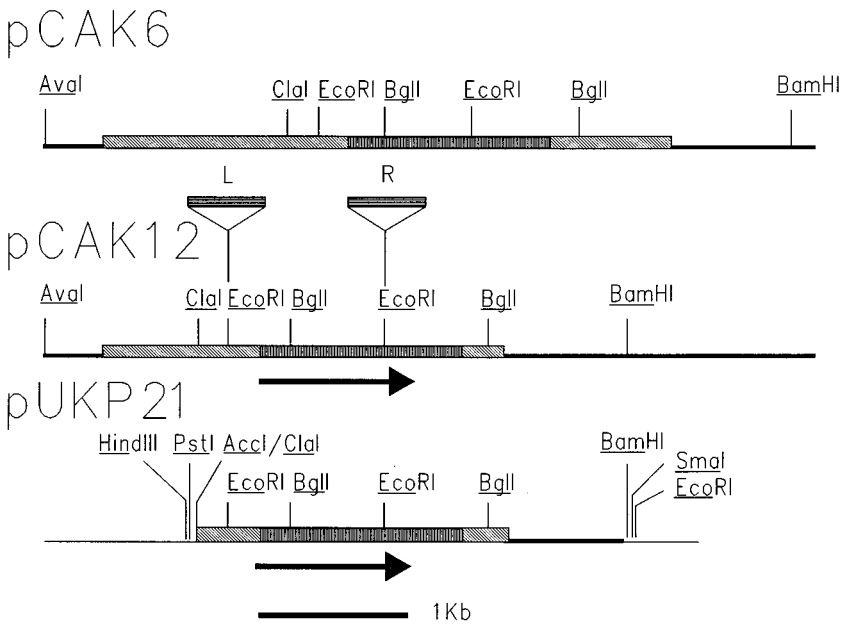

FIG. 2. Restriction map of recombinant plasmids containing the gonococcal *kat* gene. Hatching denotes cloned chromosomal DNA of *N. gonorrhoeae*, and vertical hatching marks the extent of the *kat* gene within the cloned sequences. The heavy lines are pCa2-derived sequences; the thin lines indicate pUC8 sequences. The rectangles designated L (Kat^-) and R (Kat^+) show the sites of insertion of the Spc^r Omega fragment. The arrow shows the deduced direction of transcription of *kat*.

and 87% of the transformants also acquired the Kat^+ phenotype. In contrast, pCa2 DNA purified from 2374 produced only Pen^r Kat^- transformants when used to transform 23744. When transformants were screened for plasmid content, Pen^r Kat^- colonies were found to contain deleted forms of pCaK12 or pCaK6 whereas Kat^+ transformants contained plasmids of the same size as pCaK12 or pCaK6. The preparations of pCaK6 and pCaK12 DNA used above gave rise to Pen^r DH5aMCR transformants that appeared to uniformly exhibit high levels of catalase activity when screened with 3.0% H_2O_2. Attempts to introduce pCaK6 and pCaK12 into the *E. coli* $KatE^-$ $KatG^-$ mutants UM2 and UMR1 to demonstrate complementation were unsuccessful, regardless of whether the plasmid DNA was obtained from 23744, DH5aMCR, or DH5a. However, these results appeared to be related to the vector, because pCa2 purified from the same bacterial hosts transformed neither UM2 nor UMR1 to Pen^r, but both were transformed efficiently by pUC8 and pGB2.

Restriction analysis of recombinant plasmids. Restriction analysis indicated that pCaK6 (15.7 kb) contained an insert of approximately 4.5 kb and appeared to retain all pCa2 sequences, whereas pCaK12 (14.5 kb) contained a 3.3-kb insert. The inserts in both plasmids contained cleavage sites for *Cla*I, *Bgl*I, and *Eco*RI at similar positions (Fig. 2). The sequences of both inserts bounded by two *Bgl*I sites located 1.8 kb apart were homologous with one another (Fig. 3A) and hybridized to Southern transfers of restriction digests of chromosomal DNA from strain 2821 (Fig. 3B).

The evidence of overlapping sequences in the inserts above and the estimated size of the gonococcal catalase protein (1) suggested that the putative *kat* gene responsible for the strong Kat^+ phenotype was located between the *Cla*I site and the right end of the insert at the fused *Rsa*I-*Pvu*II junction of pCaK12 and included at least one of the *Eco*RI sites (Fig. 2). Insertion mutagenesis with the Omega fragment as described in Materials and Methods confirmed this hypothesis. Plasmids pCaK12-1 and pCak12-3 contained Omega in the rightward *Eco*RI site and produced only small amounts of catalase, while pCaK12-2 and pCaK12-4, with Omega in the leftward *Eco*RI site, produced high levels of catalase characteristic of DH5a MCR/pCaK12. Insertion mutagenesis of *kat* was confirmed by transforming the gonococcal strain 2374 to Spc^r with pCaK12-1 and pCaK12-2 (from DH5aMCR). One hundred tested Spc^r transformants obtained with pCaK12-1 DNA were Kat^-, while 100 transformants obtained with pCaK12-2 were uniformly Kat^+. The results of quantitative assays for catalase activity in two transformants are presented in Table 2. Both 23752 and 23753 were exposed in vitro to H_2O_2 to determine sensitivity (Table 3).

Subcloning of *kat* from pCaK12 and complementation of a Kat mutation. The *Cla*I-*Bam*HI fragment of pCaK12 was ligated into pUC8 cleaved with *Acc*I and *Bam*HI. Pen^r Lac^- transformants of DH5aMCR produced large amounts of catalase and contained a 6.6-kb plasmid. When these plasmids were transformed into UMR1, which lacks both hydroperoxidases found in *E. coli*, they gave rise to Pen^r transformants that produced high levels of catalase activity and likewise contained 6.6-kb plasmids, confirming that the 6.6-kb plasmids carried a *kat* gene subcloned from pCaK12. To determine if the *lac* promoter affected the expression of the cloned *kat* gene, the region between the *Bam*HI and *Pst*I sites of pUKP21 was subcloned into pGB2, which contained the polylinker of pUC8 but lacked both the truncated *lacZ* gene and the *lac* promoter (8). The resulting plasmid, pGB2kat1, continued to efficiently express catalase (Table 2), indicating that the insert present in both pUKP21 and pGB2kat1 contained both the *kat* structural gene and promoter.

Sequencing of the gonococcal *kat* gene. Figure 4 gives the sequence of the gonococcal *kat* gene determined as described in Materials and Methods. The 1,500-bp open reading frame codes for the gonococcal catalase. Also presented is a 341-bp region upstream of the structural gene that contains a putative

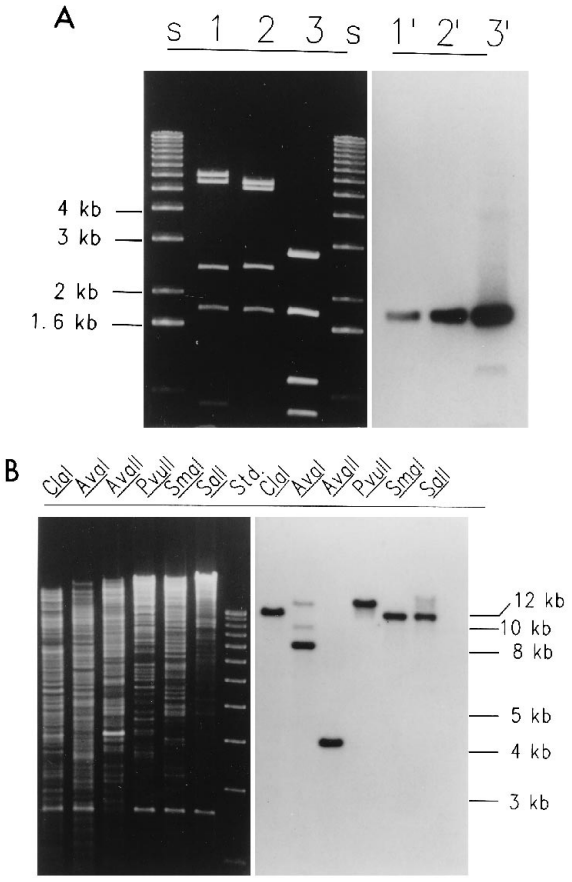

FIG. 3. Restriction digests of recombinant plasmids and homology of cloned fragments with the gonococcal chromosome. (A) pCaK6, pCaK12, and pUKP21 were digested with *Bgl*I, and the digests were subjected to agarose gel electrophoresis. The restriction fragments were transferred to nylon membrane and hybridized with the radiolabeled 1.8-kb *Bgl*I fragment purified from a digest of pCaK12. (B) Homology of the *Bgl*I fragment of pCak6 with chromosomal DNA of *N. gonorrhoeae* 2821. Chromosomal DNA was digested with a variety of restriction enzymes. The digests were subjected to agarose gel electrophoresis, transferred to membranes, and hybridized with the 1.8-kb *Bgl*I fragment of pCaK12. Both the gel (left panel) and the corresponding Southern transfer (right panel) are shown. The standard for both gels was kilobase ladder DNA (BRL-Gibco, Grand Island, N.Y.).

Shine-Dalgarno sequence and probably contains the promoter. The size of the open reading frame indicates a deduced protein sequence of 500 amino acids or a molecular mass of 56.7 kDa.

Analysis of catalase produced from recombinant plasmids in *E. coli*. Sonic extracts of cultures of UMR1 that contained either the vectors pUC8 and pGB2 or the corresponding recombinant plasmids pUKP21 and pGB2kat1 were assayed quantitatively for levels of catalase activity. The introduction of either recombinant plasmid into UMR1 resulted in the production of high levels of catalase activity by that strain (Table 2). Extracts from both *E. coli* and *N. gonorrhoeae* were also subjected to polyacrylamide gel electrophoresis under nondenaturing conditions. When stained for enzymatic activity, the gels showed the appearance of a single catalase activity with migration properties similar to those of the catalase of *N. gonorrhoeae* 2374 (Fig. 5).

To determine if expression of the gonococcal *kat* gene was influenced by *katF* (*rpoS*), pUKP21 and pGB2kat1 were transformed individually into the *katF* mutant UM56-64. Both pUKP21 and pGB2kat1 continued to express high levels of catalase activity in the absence of a functional *katF* gene (Table 2).

TABLE 2. Catalase activity of bacterial strains

Strain/plasmid	Phenotype	Catalase activity (U/mg of protein)
2374	Kat$^+$	480
23744	Kat$^-$	<0.1
23752	Kat$^+$	533
23753	Kat$^-$	<0.1
UMR1/pUC8	Kat$^-$	<0.1
UMR1/pUKP21	Kat$^+$	1,098
UMR1/pUKP21-1	Kat^{+a}	25
UMR1/pUKP21-2	Kat^{+a}	36
UMR1/pGB2	Kat$^-$	<0.1
UMR1/pGB2Kat1	Kat$^+$	1,021
UM56-64	Kat$^-$	2
UM56-64/pUKP21	Kat$^+$	1,128
UM56-64/pGB2Kat1	Kat$^+$	984

[a] Spontaneous variants of pUKP21 that express reduced levels of catalase activity.

Instability of catalase production in *E. coli*. Colonies that produced relatively little or no catalase activity were encountered at unexpectedly high frequency (8 to 12%) during growth of two cultures of UMR1 transformed independently with pUKP21. Ten colonies that produced no detectable enzyme contained plasmids ranging from 2.3 to 2.9 kb in size (data not shown). Two independently derived colonies that produced low levels of catalase activity (Table 2) harbored plasmids that contained small inserts in the 1.5-kb *Eco*RI fragment of pUKP21 (Fig. 6). The electrophoretic mobility of the catalase produced by UMR1/pUKP21-1 and UMR1/pUKP21-2 did not differ detectably from the mobility of catalase produced by UMR1/pUKP21 (Fig. 7), which indicated that the protein itself was unaffected by the insertion. The sequence data for pUKP21-1 and pUKP21-2 confirmed that a copy of IS*1* was inserted 77 bp upstream of the Shine-Dalgarno sequence in both plasmids (Fig. 8). Both insertions left the putative ribosome-binding site intact and situated adjacent to the *kat* gene. However, the insertions separated the structural gene from most of the region located 5′ to the structural gene. The drastic reduction in the amount of catalase activity expressed by pUKP21-1 and pUKP21-2 indicated that this region was important for the expression of catalase.

DISCUSSION

Efforts to clone the *kat* gene of *N. gonorrhoeae* by conventional methods were uniformly unsuccessful. The reasons for the difficulty were unclear and unexpected, because *kat* genes

TABLE 3. Sensitivity of Kat$^+$ and Kat$^-$ strains of *N. gonorrhoeae* to 10 mM hydrogen peroxide

Strain	Phenotype	% Survival[a]
2374	Kat$^+$	42.1 ± 6.2
23744	Kat$^-$	0.2 ± 8.5
23752	Kat$^+$ Spc^{rb}	78.0 ± 5.1
23753	Kat$^-$ Spc^{rc}	1.4 ± 9.0

[a] Bacteria were exposed to 10 mM H_2O_2 for 10 min, and the numbers of viable cells were determined as described previously (15).
[b] Strain 23752 contains Omega originally inserted into pCaK12 adjacent to *kat* and is phenotypically Kat$^+$.
[c] Strain 23753 contains Omega originally inserted into pCaK12 *kat* (see the text) and is phenotypically Kat$^-$.

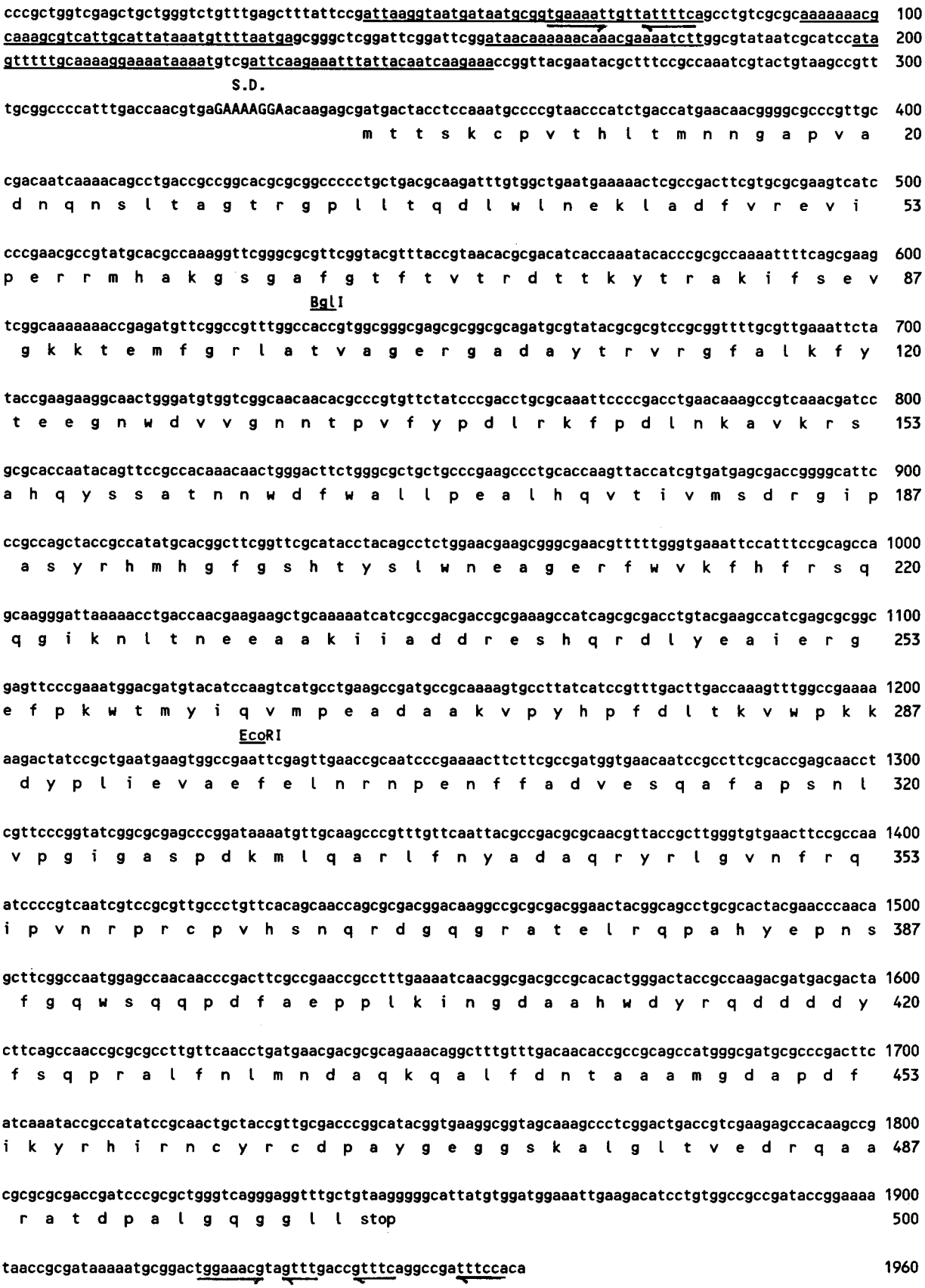

FIG. 4. Sequence of the *kat* gene of *N. gonorrhoeae* 2821 and the deduced amino acid sequence of the polypeptide. The AT-rich regions mentioned in the text are underlined. The arrows indicate regions that appear capable of base pair formation to give stem-loop structures. The putative Shine-Dalgarno sequence (S.D.) is presented in capital letters.

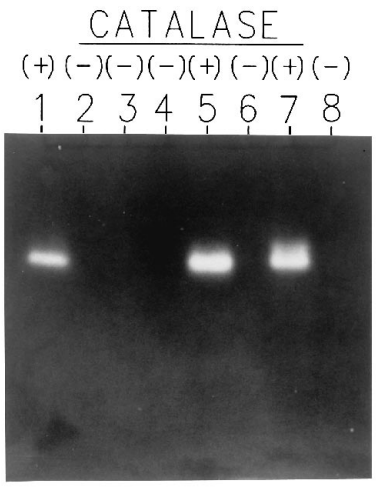

FIG. 5. Polyacrylamide gel electrophoresis of catalase expressed from the cloned *kat* gene of *N. gonorrhoeae*. After electrophoresis, the gel was stained for catalase activity as described previously (15). The samples and amounts of protein used are as follows: lane 1, 2374, 11 μg; lane 2, 23744, 10 μg; lane 3, 23753, 20 μg; lane 4, UMR1, 20 μg; lane 5, UMR1/pUKP21, 1 μg; lane 6, UMR1/pUC8, 10 μg; lane 7, UMR1/pGB2kat1, 1 μg; lane 8, UMR1/pGB2, 20 μg.

from a variety of sources have been successfully cloned. As an alternative, an attempt was made to clone the gonococcal *kat* gene into pCa2, a plasmid derived from a 7.2-kb β-lactamase plasmid, and the 4.2-kb gonococcal cryptic plasmid with a Kat^- mutant of *N. gonorrhoeae* as the host strain. Unlike the 7.2-kb β-lactamase plasmid, pCa2 efficiently transformed *N. gonorrhoeae* 2374 and its Kat^- derivative 23744 to Pen^r (37). The initial absence of plasmids from strain 23744 simplified the process of screening transformants for recombinant plasmids. The use of pCa2 and strain 23744 resulted in a total of 17 Pen^r Kat^+ colonies. Five of these colonies contained plasmids indistinguishable in size from the vector pCa2 and probably resulted either from unlinked cotransformation of the vector and gonococcal DNA fragments bearing the *kat* gene into 23744 or from recombination of a *kat* containing recombinant plasmid with the recipient chromosome. The remaining 12

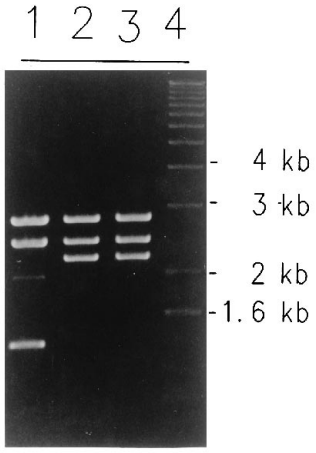

FIG. 6. Restriction analysis of plasmids derived from pUKP21 that express reduced levels of catalase. Plasmids that express reduced levels of catalase in UMR1 were purified and digested with *Eco*RI, and the digests were subjected to agarose gel electrophoresis. Lanes: 1, pUKP21; 2, pUKP21-1; 3, pUKP21-2; 4, standard.

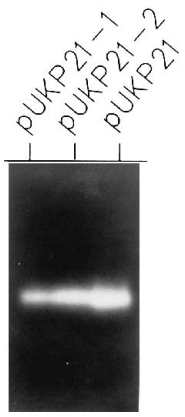

FIG. 7. Polyacrylamide gel electrophoresis of catalase from recombinant plasmids that express low levels of gonococcal catalase; pUKP21-1, 20 μg of protein; pUKP21-2, 20 μg of protein; pUKP21, 1 μg of protein.

colonies contained plasmids substantially larger than pCa2 and were considered to contain potential clones. Selection for penicillin resistance conferred by the pCa2 vector appeared to give adequate numbers of recombinant plasmids from a ligation reaction to ensure the cloning of a single gene, *kat*, without any selective pressure for *kat*. Background transformation of strain 23744 to Kat^+ with donor chromosomal DNA was not high enough to interfere with the isolation of recombinant plasmids. Although we did not attempt to directly determine the frequency with which pCa2, containing chromosomal inserts, recombined with the gonococcal chromosome, such recombination did not appear to occur often enough to impair the isolation of clones that contained recombinant plasmids. The absence of Pen^r Kat^- transformants resulting from the transformation of DH5aMCR with pCaK6 or pCaK12 DNA purified from the Kat^- strain 23744 suggested that the mutation in *kat* was rarely transferred by recombination from the chromosome of 23744 to either pCaK6 or pCaK12.

Although pCa2, pCaK6, and pCaK12 all transformed 23744 to Pen^r, only pCaK6 and pCaK12 caused cotransformation of 23744 to Kat^+. Plasmids pCaK6 and pCaK12 were subjected to further analysis, which showed that the inserts in both plasmids contained homologous, overlapping regions with identically placed *Cla*I, *Bgl*I, and *Eco*RI sites. The presence and location of the *kat* gene within this region of pCaK12 were confirmed by performing insertional mutagenesis of *kat* with the Omega fragment (32) and by subcloning *kat* from pCaK12 into both pUC8 and pGB2. Moreover, the insertionally mutagenized *kat* gene of pCaK12-1 was transformed into *N. gonorrhoeae* 2374 to create the Kat^- strain 23753, which was phenotypically similar to both 85-015, a spontaneous Kat^- mutant (19), and 23744, which carried the same mutation as 85-015. Insertion of Omega into a second *Eco*RI site in pCaK12 resulted in Spc^r transformants indistinguishable from 2374. In addition to confirming the cloning of *kat*, these results support our earlier conclusion that the mutation(s) present in the clinical isolate 85-015 probably inactivates the *kat* gene (19). However, the nature of the mutation in 85-015 remains to be determined.

The amino acid sequence of the catalase of *N. gonorrhoeae* showed 83% identity to that of *H. influenzae* catalase. The high level of identity was maintained even in the carboxy-terminal region, which varies considerably in length and composition among catalase polypeptides (4). Consistent with the high degree of identity between the two proteins, the coding sequence of the gonococcal *kat* gene showed 74.8% homology with the

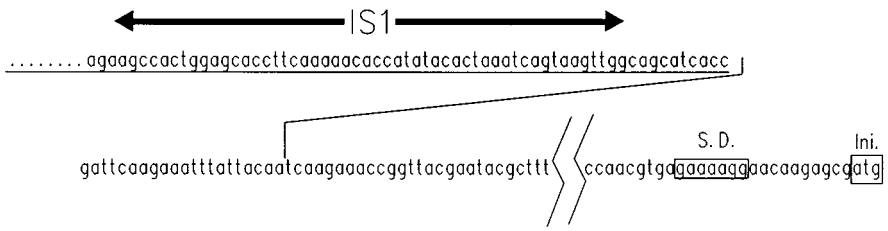

FIG. 8. Location of the insertions in pUKP21-1 and pUKP21-2. A primer homologous to sequences located in the 5′ region of the gonococcal *kat* gene was used for dideoxynucleotide sequencing of the region upstream of the structural gene. The diagram shows the site of the junction between the gonococcal DNA in pUKP21-1 and pUKP21-2 and the 3′ end of the IS*1* copy.

coding sequence of *hktE* (4). This value is compared with 65% homology between the *recA* structural genes of the two organisms (13, 42), 61% homology between the structural genes coding for immunoglobulin A proteases (an unusual protease activity shared by both organisms) (30, 31), and 69% homology between the structural gene encoding the major iron-binding protein of *N. gonorrhoeae* and the *hitA* structural gene of *H. influenzae* (3, 34). However, the sequences flanking the structural genes differed considerably in terms of both sequence and organization. While a putative Shine-Dalgarno sequence was situated 8 bp in advance of an acceptable initiation codon, no good candidates with appropriate sequence and spacing corresponding to −10 and −35 sequences were found. Unlike *hktE*, the gonococcal *kat* gene was not preceded by any sequence that exhibited significant homology with the OxyR-binding consensus sequence (4). The region of the gonococcal gene 67 bp in advance of the Shine-Dalgarno sequence was unusual for its composition and organization. It was composed of five distinct stretches with high A+T content, each of which was bounded by short intervals with higher G+C content (Fig. 4). The first two of these AT-rich regions were 39 and 43 bp long and contained 74 and 72% A+T, respectively. The remaining three regions ranged from 25 to 28 bp long and had A+T contents ranging from 80 to 82%. All five regions were characterized by extensive runs of A residues. The first of these regions contained an 18-bp sequence capable of forming a stem-loop structure, preceded by a series of five potential stop codons, which suggested that another open reading frame with strong transcriptional and translational stops was located immediately upstream of *kat*. The sequences on either side of the entire AT-rich region were more typical of the genomic DNA of *N. gonorrhoeae* (38). This AT-rich region may constitute the promoter for gonococcal catalase, and its unusual composition and organization may be related both to the efficiency with which the gene is transcribed and to the relative insensitivity of expression to induction by hydrogen peroxide (41). Compared with the *hktE* gene, the gonococcal gene also lacked a clearly defined termination sequence characterized by long inverted repeats but did possess a set of short sequences capable of base pairing over four to six nucleotide residues.

The gonococcal *kat* gene was efficiently expressed in *E. coli*, with no pronounced difference in the levels of specific activity regardless of whether the *kat* gene was carried on pUKP21, which possesses the efficient *lac* promoter, or on pGB2kat1, which does not have the *lac* promoter adjacent to the polylinker region (8). In addition to being unaffected by the presence of the *lac* promoter, the amount of catalase activity was essentially unaffected by the wide disparity in the numbers of plasmid and *kat* gene copies present in UMR1 containing pUKP21 as opposed to UMR1 containing pGB2kat1 (8). These results, showing that catalase levels were independent of gene dosage, suggested that transcription and subsequent translation of *kat* were not limiting factors for the expression of the gene in *E. coli*; thus, it would appear that the *kat* promoter must be extremely efficient and the gene must not be stringently regulated. In fact, the levels of catalase expressed from pGB2kat1, a low-copy plasmid (8), corresponded well to the levels of catalase detected for the chromosomally located *kat* gene in *N. gonorrhoeae* when the slightly elevated gene dosage for pGB2kat1 was taken into account. It was therefore not surprising that the cloned gonococcal *kat* gene, when resident in *E. coli*, did not require the *katF* (*rpoS*) gene product for expression (22, 27) as does the *katE* gene of *E. coli*. The extremely high level of catalase produced by *N. gonorrhoeae*, compared with other, related pathogens in which catalase production has been studied, appears to be a direct property of the gene and its promoter and not the result of multiple genetic factors within the gonococcus. Survival on human genital mucosa may require levels of catalase in *N. gonorrhoeae* that are not required for *Neisseria meningitidis* or *Haemophilus influenzae* to colonize and survive on the mucosa of the nasopharynx. The role of catalase in the biology of these last two organisms probably differs from that in the biology of the gonococcus. The striking differences between the promoter regions of the gonococcal *kat* gene and *hktE*, two otherwise highly related genes, and the differences in the levels and regulation of expression between them support such a conclusion.

Given the relatively efficient expression of catalase, the frequency with which colonies of UMR1/pUKP21 failing to produce catalase or producing low levels of catalase were isolated was unexpectedly high. Colonies that failed to produce catalase simply contained plasmids that had sustained large deletions. Of greater interest were the isolates that produced reduced levels of catalase and contained plasmids with insertions of IS*1* located 77 bp 5′ to the putative ribosome-binding site. These insertions of IS*1* were essentially spontaneous regulatory mutations of the *kat* gene, and they confirmed the importance of the AT-rich 5′ regions for high-level expression of catalase.

In addition to cloning the gonococcal *kat* gene and presenting evidence to support the conclusions reached in our earlier characterization of a spontaneous Kat$^-$ isolate of *N. gonorrhoeae*, we have demonstrated that it is possible to directly clone gonococcal genes into genetically appropriate strains of *N. gonorrhoeae*. Hypothetically, any gene which can be identified phenotypically or otherwise is a candidate. This sort of direct cloning approach has some advantages over the more conventional approach. Our work indicated that very large DNA fragments of 14 to 15 kb could be introduced into *N. gonorrhoeae*, because we also cloned *kat* from a *Pvu*II digest in a 15.2-kb fragment. Because the cloned fragments are introduced directly into the gonococcus, this approach may prove valuable for cloning genes which are unlikely to be phenotypically expressed or identifiable in *E. coli* or genes whose expression may be deleterious (3, 6).

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