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Expression of an Antisense *hla* Fragment in *Staphylococcus aureus* Reduces Alpha-Toxin Production In Vitro and Attenuates Lethal Activity in a Murine Model

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Isogeneic bacterial strains that differ only in the production of a single microbial factor have been invaluable in studying the pathogenesis of bacterial infections. The targeted, intentional inactivation of a gene encoding a potential virulence determinant generally requires homologous recombination to replace the gene with an inactivated allele. To determine whether the insertion and expression of a fragment of a bacterial gene in an antisense orientation could be used as a rapid alternative to allelic inactivation for producing paired isogeneic isolates, we inverted a 600-bp fragment of the *Staphylococcus aureus* gene encoding alpha-toxin, *hla*, behind its native promoter on an *Escherichia coli-S. aureus* shuttle vector. A transformant of an *S. aureus* strain carrying the antisense *hla* fragment produced antisense *hla* RNA and made 16-fold less alpha-toxin than either its parent or an isogeneic transformant containing vector DNA without *hla*. Also, intraperitoneal injection of 1.5×10^9 CFU of the antisense *hla*-containing transformant was significantly less lethal in a murine model than that of the parent (1 of 10 versus 7 of 10 mice expired [$P < 0.02$]) or the transformant without *hla* (1 of 10 versus 7 of 7 mice expired [$P < 0.001$]). We conclude that the expression of a fragment of *hla* in an antisense orientation in *S. aureus* on a plasmid vector reduces alpha-toxin production and the lethal activity of the strain in a murine model. The antisense strategy for creating isogeneic strains of bacteria may facilitate molecular investigations into the pathogenesis of infection. It also may be useful in creating novel live-attenuated strains of bacteria for use as vaccine candidates.

Recently, there has been impressive success in eukaryotic biotechnology related to the cloning and expression of antisense DNA. This strategy has produced transgenic plants and animals that have been rendered resistant to specific viral pathogens by incorporating antisense viral genomic DNA fragments into the eukaryotic genome (9, 31). In addition, tomatoes with a longer shelf life because of the reduced production of a degradative enzyme, polygalacturonase, have been constructed by using antisense techniques (35). Currently under investigation is the use of antisense oligonucleotides with enhanced stability and improved ability to permeate membranes as novel and, it is hoped, nontoxic therapies for treating neoplasms (16, 23, 32) and a variety of infectious agents ranging from the human immunodeficiency virus to *Leishmania* spp. (7, 30, 39–41). These developments, along with the natural occurrence of antisense DNA as a regulator of plasmid copy number in some bacterial species (14, 37), led us to wonder whether antisense techniques could be used in bacteria as a tool to reduce the production of specific microbial products implicated in pathogenicity and as an alternative to insertional mutagenesis (13, 18, 26) for creating isogeneic strains. In this study, we investigated whether the cloning and expression of an antisense fragment of the gene encoding *S. aureus* alpha-toxin (*hla*) can be used to reduce the production of this lethal toxin and whether the resulting isogeneic strain exhibits diminished lethal activity in a murine model.

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MATERIALS AND METHODS

Materials and media. Restriction endonucleases were purchased from Promega (Madison, Wis.) and United States Biochemical Corp. (Cleveland, Ohio). *Escherichia coli* strains were cultivated in LB media (34). *S. aureus* strains were propagated in tryptic soy broth and agar (Difco Laboratories, Detroit, Mich.) or LB media. Chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) and erythromycin (United States Biochemical Corp.) were used for selection of *E. coli* and *S. aureus* transformants, respectively.

Bacterial strains, plasmids, and growth characteristics. The major plasmids and strains used or constructed during this investigation are listed in Table 1. Transformation of *E. coli* was performed by using strain DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) (15). Protoplast transformation (28) was used to transfer plasmids harvested from *E. coli* into *S. aureus* RN4220, after which they could be transformed into strain DK2076. *S. aureus* transformants were grown in media containing 10 μg of erythromycin per ml to protect against plasmid loss unless otherwise indicated, whereas the parent strain DK2076 was erythromycin susceptible and always grown in media without erythromycin. Growth rates of the isogeneic bacterial strains were compared by monitoring A_{600} over time. Total protein production was compared by the method of Bradford (6) with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, Calif.). In addition, each of the isogeneic strains was grown in LB broth to an A_{600} of 1.5 and the protein banding patterns of 5 ml of their culture supernatants which had been precipitated with trichloroacetic acid and electrophoresed on a 15% polyacrylamide gel were compared.

Northern hybridization. Single-stranded DNA oligonucleotides based on a portion of *hla* within the 600-bp *Kpn*I fragment were constructed by the Vanderbilt Molecular Physiology CORE laboratory to specifically hybridize with either sense (5′-AACTGTACCTTAAAGGCTGAAGGCCAGGCTAAACCACTTTTGTTAGCACC-3′) or antisense (5′-GGTGCTAACAAAAGTGGTTTAGCCTGGCCTTCAGCCTTTAAGGTACAGTT-3′) RNA. Ten nanograms of each oligonucleotide was end labeled with [γ-^{32}P]dATP (Du Pont NEN Research Products, Boston, Mass.) by using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) for 90 min at 37°C. The labeled oligonucleotide was purified on a Select G-25 column (5Prime→3Prime, Inc., Boulder, Colo.).

To isolate mRNA from strains of *S. aureus*, starter cultures were prepared by inoculating 10 ml of broth with each isolate and incubating it overnight at 37°C. Each culture was diluted with fresh broth to an A_{600} of 0.3, aliquoted as 10-ml portions into 50-ml conical tubes, and reincubated on an incubator-rotary shaker

TABLE 1. Strains and plasmids used in this study

Plasmids and strains	Relevant characteristic(s)	Source; reference
Plasmids		
pDU1150	pBR322 with cloned *hla* from strain Wood 46 on 3.0-kb *Hin*dIII-*Eco*RI fragment	T. J. Foster[a]; 20
pVK100	*E. coli-S. aureus* shuttle vector made by ligating *E. coli* phagemid pBCSK+ (*Sac*I digest) and staphylococcal plasmid pE194 (*Sal*I digest) together	pVK100, this study; pBCSK+, Stratagene[b]; pE194, K. G. H. Dyke[c]; 17
pVK150	pVK100 with 3-kb fragment from pDU1150 containing *hla* in sense orientation	This study
pVK151	pVK100 with 3-kb fragment from pDU1150 with 600-bp *Kpn*I intragenic portion of *hla* cut and religated in antisense orientation behind *hla* promoter	This study
Strains		
RN4220	Relatively avirulent *S. aureus* strain derived from chemical mutagenesis that accepts DNA from *E. coli* and is useful as intermediate host for cloning of DNA into other staphylococcal strains	R. P. Novick[d]; 27
DK2076	Phage group 94/96 strain, lacks plasmids	Clinical isolate

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[b] La Jolla, Calif.
[c] Oxford University.
[d] Public Health Research Institute, New York, N.Y.

at 150 rpm (New Brunswick Scientific Co., New Brunswick, N.J.) until an A_{600} of 1.5 was achieved. Whereas the erythromycin-susceptible parent strain was grown without erythromycin, the three transformants were grown in media containing 10 μg of erythromycin per ml and took 1 h longer to reach the desired A_{600} than the parent strain (3.5 h rather than 2.5 h). The cells were centrifuged at 3,000 × *g* for 10 min at 4°C, and total cellular RNA was isolated and purified with the Qiagen RNeasy protocol and column (Qiagen, Inc., Chatsworth, Calif.).

Samples from each strain applied to lanes of a gel were adjusted by measuring A_{260} to verify a total cellular RNA content of 5 μg. The samples were electrophoresed through a 1.5% agarose-formaldehyde gel containing ethidium bromide and transferred to a Hybond-N nylon membrane (Amersham Life Science Products, Arlington Heights, Ill.) with capillary methods by using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate, pH 7.0). After drying of the membrane, the RNAs were cross-linked by UV irradiation. Gels and membranes were prepared in duplicate for use with the two probes.

The duplicate membranes were prehybridized for 4 h at 42°C in a 0.15-ml/cm^2 buffer solution containing 1× Denhardt solution, 6× SSC, 100 μg of salmon sperm per ml, and 0.5% sodium dodecyl sulfate in 20 mM $NaHPO_4$, pH 7.4. Hybridization was performed for 18 to 24 h at 42°C in the same buffer containing 2 ng of the ^{32}P-labeled probe for either sense or antisense RNA per ml. The membranes were washed, and DNA-RNA hybridization was detected with X-Omat AR film (Eastman Kodak Company, Rochester, N.Y.).

Quantification of alpha-toxin production. Immune serum for Western blotting was prepared with a New Zealand White rabbit (Myrtle's Rabbitry, Columbia, Tenn.) by using purified alpha-toxin containing a leucine-for-histidine substitution at amino acid 35 (H35L toxin) as the immunogen (24, 25). The H35L toxin is nontoxic while retaining immunogenicity and was purified from culture supernatants of strain DU1090(pH35L) by using the controlled-pore glass method of Cassidy and Harshman (8). Samples for Western blot assays were supernatants of 18-h tryptic soy broth cultures of the isogeneic strains grown without erythromycin with comparable total protein concentrations that had been diluted 5-, 20-, and 80-fold. The samples were applied to a sodium dodecyl sulfate–12% polyacrylamide gel. The gel was blotted onto a nitrocellulose membrane in Tris-glycine-20% methanol buffer and blocked for 2 h at 25°C with 0.5% Tween 20 in Tris-buffered saline (10 mM Tris, 500 mM NaCl, pH 8.0) (TSBB). The membrane was incubated at room temperature with a 1:500 dilution of anti-H35L serum, washed three times in TSBB-Tween, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Boehringer Mannheim, Indianapolis, Ind.). At the end of the second incubation, the membrane was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Murine intraperitoneal injection studies. The in vivo experiments were approved by the institutional committee for animal care. Male NIH Swiss mice (Harlan Sprague-Dawley, Indianapolis, Ind.), weighing 20 to 24 g each, were injected intraperitoneally (i.p.) with 0.5 ml of a suspension of *S. aureus* DK2076 or one of its isogeneic derivatives by using 25-gauge needles and tuberculin syringes. Buprenorphine, at a dose of 2 mg/kg of body weight, was administered intramuscularly at the time of i.p. injection to minimize suffering. The bacterial suspension was prepared by inoculating 50 μl of the 18-h tryptic soy broth cultures prepared as described above into fresh tryptic soy broth without erythromycin. The subcultures were incubated at 37°C for 1.5 to 2 h until the A_{600} was 0.2, after which the bacterial cells were pelleted by centrifugation at 3,000 × *g* for 10 min, washed three times with 0.1 M phosphate-buffered saline (pH 7.0), and adjusted spectrophotometrically to a density of approximately 3×10^9 CFU/ml. Backcounts were performed to determine the precise colony count in each inoculum. Hemolytic assays were performed as previously described (3, 24, 25) and demonstrated that each inoculum had an alpha-toxin titer of less than 1 hemolytic unit at the time of injection. Death was used as the endpoint, and the time from i.p. injection until death was recorded. Mice that did not expire from the bacterial inoculation were sacrificed at 6 days postinjection by cervical dislocation. The peritoneum was opened, and the abdomen was examined for the presence of an abscess. In addition, the spleen and kidneys from each animal were harvested, homogenized, and cultured on sheep blood agar. The two-tailed Fisher exact test was used to determine the statistical significance of differences in the number of mice in each set that had expired. In addition, Kaplan-Meier plots (19) were analyzed with NCSS software (version 5.5; Hintze JL, Kaysville, Utah) and statistical significance was determined by using the Peto/Wilcoxon test.

RESULTS

Construction of isogeneic *S. aureus* strains. To determine if the expression of a fragment of *hla* in an antisense orientation with respect to that of the endogenous chromosomal gene could be used to create isolates of *S. aureus* with diminished production of alpha-toxin, we first inverted a 600-bp *Kpn*I intragenic fragment of cloned *hla* on *E. coli* plasmid pDU1150 in an antisense direction behind its native promoter (Fig. 1). The orientation of the 600-bp fragment was determined by observing DNA fragment sizes following endonuclease digestion with *Sca*I (6.1 and 1.5 kb for the antisense construct versus 6.5 and 1.1 kb for the sense construct). A 3.0-kb fragment containing the antisense construct was ligated into an *E. coli-S. aureus* shuttle vector, pVK100, to produce pVK151, which was transformed into *E. coli* DH5α and then into *S. aureus* RN4220, which is capable of accepting DNA directly from *E. coli*. Subsequently, pVK151 harvested from RN4220 was transformed into the virulent strain *S. aureus* DK2076. Control plasmids were similarly transformed into the same recipient strains and included (i) the shuttle vector lacking the 3.0-kb region containing *hla* (pVK100) and (ii) the shuttle vector into

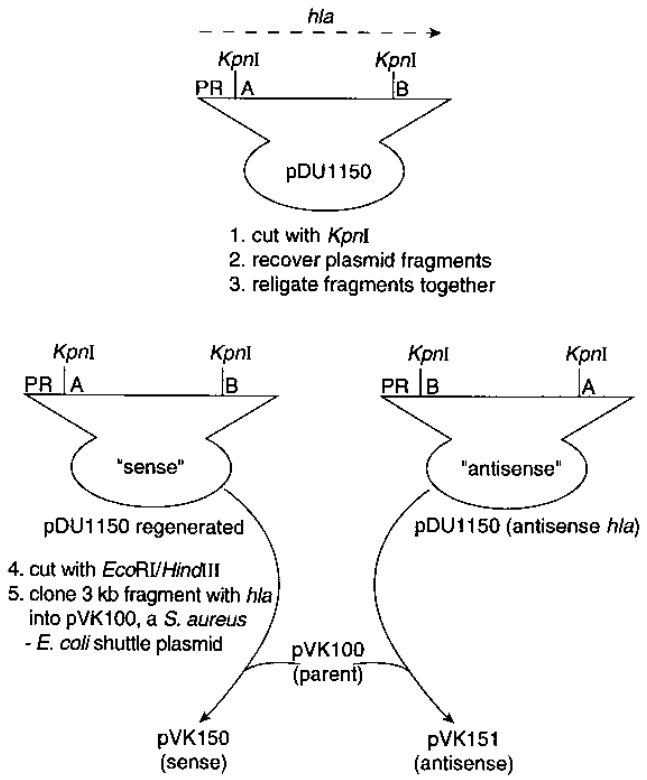

FIG. 1. Construction of isogenic mutants of *S. aureus* bearing pVK100 with *hla* in the sense and antisense orientations. Cloned *hla* in pDU1150 was digested with *Kpn*I. As *hla* has two *Kpn*I sites, when the cut fragments of the plasmid reassembled, some did so with *hla* in its original sense orientation, whereas in others the intragenic portion of *hla* had inverted to an antisense orientation behind the promoter. Next, the 3.0-kb *Hin*dIII-*Eco*RI fragment containing either the sense or antisense *hla* gene was inserted into the *E. coli-S. aureus* shuttle vector pVK100 to form pVK150 and pVK151, respectively. These three plasmids were transformed into *S. aureus* RN4220. A and B show the orientations of the *hla* fragment relative to its promoter (PR) before and after endonuclease digestion.

which the 3.0-kb fragment had been cloned with *hla* in its normal sense orientation (pVK150).

In vitro characterization of isogeneic strains. In the absence of erythromycin, transformants of *S. aureus* DK2076 containing pVK100, pVK150, and pVK151 exhibited growth kinetics and total protein production indistinguishable from those of the parent strain. Whereas erythromycin was needed for the transformants to maintain the plasmids over multiple generations, it caused a 30 to 50% reduction in the growth rate compared with strains grown without erythromycin and reduced alpha-toxin production. Accordingly, for studies involving Western hybridization and in vivo lethal activity, the transformants were grown with erythromycin until the final subculture, which was grown without erythromycin.

Northern hybridization using a single-stranded probe specific for sense *hla* RNA showed quantities of mRNA comparable to that of the parent strain in DK2076(pVK100), relatively increased in DK2076(pVK150), and relatively reduced in DK2076(pVK151) (Fig. 2). Northern hybridization using a single-stranded probe specific for antisense *hla* RNA confirmed that it was produced only by DK2076(pVK151).

A 16-fold reduction in the amount of alpha-toxin in the culture supernatant of DK2076(pVK151) compared with the supernatants of DK2076 and DK2076(pVK100) was observed with Western hybridization (Fig. 3). In contrast, DK2076

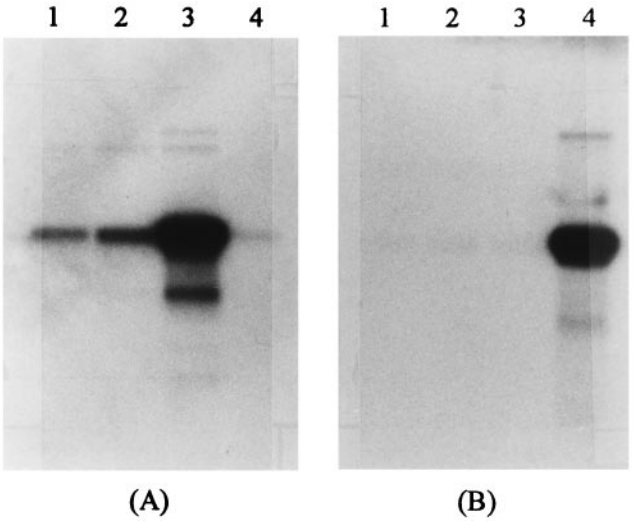

FIG. 2. Northern hybridization using single-stranded DNA oligonucleotide probes constructed to hybridize specifically with *hla* RNA in either a sense (A) or an antisense (B) orientation. Lanes: 1, DK2076, parent strain; 2, DK2076(pVK100), shuttle plasmid without *hla*; 3, DK2076(pVK150), shuttle plasmid with sense *hla*; 4, DK2076(pVK151), shuttle plasmid with antisense *hla*

(pVK150) produced the greatest amount of alpha-toxin, probably reflecting a gene dosage effect. Coomassie blue-stained polyacrylamide gels of trichloroacetic acid-precipitated culture supernatants of the four isogeneic strains showed changes in the intensity of the protein band corresponding to alpha-toxin comparable to that demonstrated by Western hybridization. There were no other detectable qualitative or quantitative differences among the protein banding patterns of the four strains.

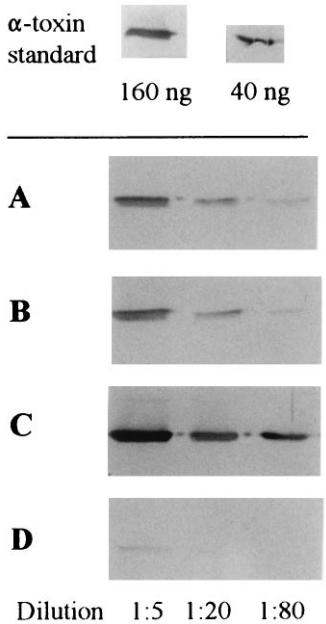

FIG. 3. Western blots of alpha-toxins produced by strain DK2076 and its isogeneic derivatives. Blots: A, DK2076, parent strain; B, DK2076(pVK100), shuttle plasmid without *hla*; C, DK2076(pVK150), shuttle plasmid with sense *hla*; D, DK2076(pVK151), shuttle plasmid with antisense *hla*. Dilution factors of the 18-h broth supernatants from the isolates are indicated at the bottom.

Attenuation of the lethal activity of *S. aureus* by antisense *hla* expression. Mice injected with DK2076(pVK151) had significantly improved survival compared with mice receiving either DK2076 (1 of 10 versus 7 of 10 mice expired, respectively [$P < 0.02$]) or DK2076(pVK100) (1 of 10 versus 7 of 7 mice expired, respectively [$P < 0.001$]). DK2076(pVK150) was more rapidly lethal than the other isolates (Fig. 4; $P < 0.002$ for each comparison). As the inocula had an alpha-toxin titer of less than 1 hemolytic unit at the time of injection, the deaths were due to alpha-toxin produced in vivo following inoculation. Nine of the mice that received the antisense *hla* fragment survived the bacterial challenge. At 6 days, four appeared ill with ruffled fur and huddling behavior. Upon autopsy, two mice were identified with intraperitoneal abscesses, *S. aureus* was recovered from the kidneys of nine mice, and no organisms were cultured from the spleen of any mouse.

DISCUSSION

The goal of this investigation was to determine whether the pathogenicity of a bacterial strain could be attenuated by introducing a fragment of a gene encoding a virulence factor in an antisense expression orientation with respect to the endogenous gene. We selected *S. aureus* as the species and *hla* as the gene because of our familiarity with this system and because of the well-established role of alpha-toxin as a lethal toxin in mice (4, 5, 10, 29). We demonstrated that the production of antisense *hla* RNA diminishes the production of alpha-toxin in *S. aureus* with a corresponding reduction in lethal activity. This validates the concept that antisense methods might be used to target specific genes and investigate their role in the pathogenesis of infection.

Although early death was reduced among the mice receiving antisense *hla*, *S. aureus* was recovered from the kidneys of the nine survivors and intraperitoneal abscesses were identified in two. This was expected, as *S. aureus* makes numerous toxins and products other than alpha-toxin that are not rapidly lethal but which may contribute to its ability to cause infection (10, 12, 29).

The mechanism by which the expression of antisense gene fragments decreases the production of the protein encoded by the endogenous gene probably involves binding of complementary regions of the normal sense mRNA and the antisense RNA strand with duplex formation in a manner that blocks RNA processing and translation. However, this explanation likely understates the complexity of the interaction. In eukaryotes, RNA-RNA duplexes are substrates for an adenosine deaminase that converts adenosines to inosines, thereby inactivating both the sense and antisense strands (2, 33, 38). Another mechanism shown to play a role in the inhibitory activity of single-stranded DNA antisense oligonucleotides is the degradation of DNA-RNA hybrids by RNAse H (33, 36). Furthermore, an antigene effect has been reported for some DNA-based oligonucleotides via triple-helix formation between the oligomer and double-stranded DNA which results in the repression of gene transcription (1, 36). Whereas the quantities of alpha-toxin sense RNA (mRNA) were shown to be comparable for the parent strain and the strain bearing the vector only, they were increased and decreased for the strains bearing the sense and antisense constructs, respectively. Reduced stability of *hla* mRNA in the antisense strain due to the RNA degradative processes described above may explain why it exhibited reduced mRNA. Alternatively, the decreased signal on our Northern blot might be attributable to the alpha-toxin antisense RNA already on the blot annealing with the sense strand and blocking the probe from hybridizing.

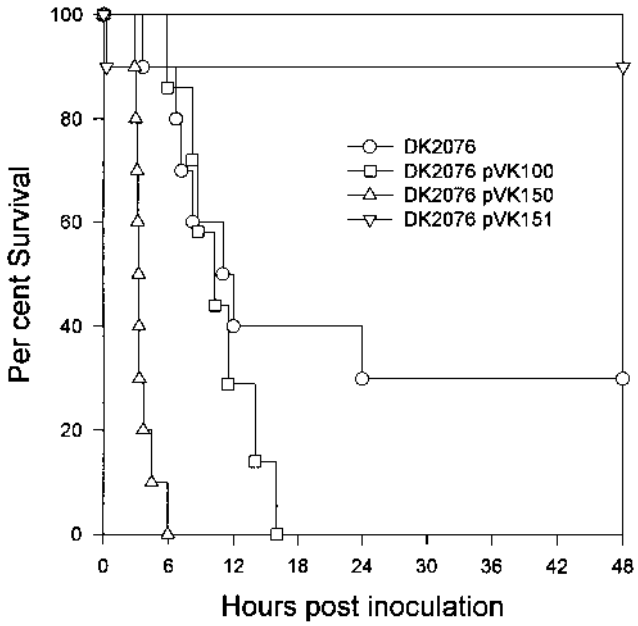

FIG. 4. Survival over time of mice receiving an i.p. injection of suspensions of strain DK2076 and its isogeneic derivatives. Each of the bacterial cell suspensions was adjusted to a standard A_{600}. Backcounts of the number of bacteria injected were as follows: DK2076, 1.1×10^9 CFU; DK2076(pVK100), 1.8×10^9 CFU; DK2076(pVK150), 1.4×10^9 CFU; DK2076(pVK151), 1.5×10^9 CFU. The single death among the mice injected with the strain containing antisense *hla* occurred within 15 min of injection, suggesting accidental injury to a major vessel or the viscera during injection rather than a toxin-mediated death.

There are several potential advantages of the antisense method for creating isogeneic strains. First, the speed and technical ease with which this strategy can be implemented deserve emphasis. With the *hla* antisense strains described here, we had viable isogeneic strains within a week of starting the work. Second, the antisense strategy should enable genes of which there are multiple copies or gene homologs to be targeted, as the expression of an antisense DNA fragment would be expected to act globally to reduce the formation of the proteins encoded by multiple homologous genes. Third, it probably will be possible to target more genes by antisense than by allelic inactivation. For genes that are required for the microbe to live, allelic inactivation is a lethal event. In contrast, the expression of an antisense DNA fragment reduces the production of the protein encoded by the gene without eliminating it completely. The reduced levels of production may be sufficient for the microbe to survive, albeit in a weakened form. This might permit the attenuation of microbes by targeting biosynthetic and other genes for which gene knockout technology causes too severe a defect. Fourth, it should be possible to insert antisense DNA fragments from multiple genes into a shuttle vector behind a common promoter. Then, the recombinant vector can be introduced into bacteria with the effect of reducing the production of multiple proteins simultaneously. In contrast, with allelic inactivation, each gene would have to be inactivated independently. Similarly, with the antisense strategy it might be possible to reduce the production of multiple proteins encoded by polycistronic genes with a single antisense DNA fragment.

Major disadvantages of the antisense strategy in bacterial species include the possibility that antisense sequence-containing vectors will be eliminated over time with reversion of the strain to the wild-type, virulent phenotype. This is of particular

concern if antisense strategies are to be used to construct novel live-attenuated vaccines. Reversion to a virulent phenotype may be of less concern if antisense DNA is inserted directly into the chromosome, e.g., into a phage integration site (21, 22) instead of a plasmid, although the advantages of copy number might be lost such that the antisense fragment is not as effective in diminishing production of the gene product as it was when it was plasmid borne. Alternatively, a vaccine strain could be an auxotroph containing a defect in a critical biosynthetic pathway. Then the defect could be complemented with a functional gene on the plasmid containing the antisense fragments. Therefore, loss of the plasmid containing the antisense fragments would be lethal for the strain and the danger of reversion to a virulent phenotype would be reduced. Ideally, the biosynthetic pathway would be unique to the microbe, e.g., the synthesis of diaminopimelate in the bacterial cell wall, such that the microbe could not obtain the essential factor from the tissues of an infected host.

Another disadvantage of the antisense strategy for creating isogeneic strains is that whereas with insertional mutagenesis it is possible to fulfill molecular Koch's postulates (11) and further verify the importance of the gene in pathogenesis by using complementation to restore pathogenicity, there is no similar way to easily confirm observations made by using antisense techniques. Ultimately, allelic inactivation or immunization studies involving the gene product might be required for confirmation of its role in infection pathogenesis.

In summary, the expression of antisense DNA can be used to downregulate the production of a microbial product and attenuate bacterial virulence in vivo. The antisense strategy for creating isogeneic strains of bacteria may facilitate molecular investigations into the pathogenesis of infection. It may also be possible to use these techniques to create novel live-attenuated strains of bacteria for use as vaccine candidates.

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