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Cloning, Complementation, and Characterization of an *rfaE* Homolog from *Neisseria gonorrhoeae*

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***Neisseria gonorrhoeae* WS1 is a spontaneous pyocin (a bacteriocin produced by *Pseudomonas aeruginosa*)-resistant mutant of *N. gonorrhoeae* FA19 that produces a truncated lipooligosaccharide (LOS) and is nontransformable. The LOS-specific mutation in WS1 was moved into a transformable background by transforming FA19 with chromosomal DNA from WS1 (generating strain JWS-1). A clone (pJCL2) capable of restoring JWS-1 to wild-type LOS expression, as detected by its acquisition of reactivity with monoclonal antibodies and by its complemented sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile, was isolated. Sequential unidirectional deletion and DNA sequence analysis of pJCL2 identified an open reading frame, designated *lsi-7*, that could complement the defect in JWS-1. Homology searches against various databases indicated that *lsi-7* had homology with several *Escherichia coli* genes involved in the phosphorylation of sugars. *lsi-7* is adjacent to the *lsi-6* gene, another gene involved in LOS biosynthesis. Complementation studies using *Salmonella typhimurium* lipopolysaccharide mutants showed *lsi-6* and *lsi-7* to be gonococcal homologs of *S. typhimurium rfaD* and *rfaE*, respectively. Reverse transcriptase PCR analysis demonstrated that *lsi-6* and *lsi-7* are part of the same transcriptional unit.**

The lipooligosaccharide (LOS) of *Neisseria gonorrhoeae* functions as an important virulence determinant in gonococcal infections. Gonococcal LOS is involved in immune evasion, attachment to epithelial cells, tissue damage to oviduct mucosa, and the stimulation of bactericidal antibodies (3, 10, 31). The role of LOS in the disease process is dependent on the production of specific LOS types or the modification of existing types during different stages of a gonococcal infection (16, 27). Modification of LOS can occur by the addition of sialic acid to the terminal galactose of the epitope recognized by the monoclonal antibody (MAb) 1B2. This structure is similar to paragloboside, a glycosphingolipid of human cells (1, 17, 27).

Gonococcal LOS consists of heterogeneous glycolipids with up to three oligosaccharide chains branching from two internal heptose residues. LOS has a single, nonrepeating α-oligosaccharide extending from heptose I. Two additional chains, the β- and γ-oligosaccharide chains, extend from heptose II (8, 13). Inter- and intrastrain variations result from the production of antigenically distinct LOS types which differ in length (α-oligosaccharide) or presence (β-oligosaccharide) of LOS components, their relative concentrations, and their number (2, 11, 15, 28). LOS variation in the gonococcus can be regulated by changes in a poly(G) tract within *lsi-2*, altering the length of the α-oligosaccharide chain in *N. gonorrhoeae* (5, 9).

A variety of strategies to identify genes involved in LOS biosynthesis have been used. LOS biosynthetic genes have been cloned by complementing pyocin (a bacteriocin produced by *Pseudomonas aeruginosa*)-resistant mutants of *N. gonorrhoeae*, identified on the basis of their ability to alter the lipopolysaccharide (LPS) of *Escherichia coli* or by complementing specific genes involved in LPS synthesis (6, 19–21, 23, 34). Other LOS biosynthetic genes have been identified on the basis of their ability to alter LOS expression in transformation assays (5) or through DNA sequence analysis of biologically interesting clones (9).

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In this work we report the cloning and characterization of *lsi-7*, a genetic locus capable of complementing the pyocin-derived LOS mutant WS1, a strain that produces a truncated LOS void of heptose. Complementation analysis using *Salmonella typhimurium* LPS mutants served to identify the in vivo function of *lsi-7* as a homolog of *rfaE.*

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmid curing, and plasmid manipulations. *N. gonorrhoeae* WS1 and FA19 were kindly provided by William Shafer (Emory University School of Medicine, Atlanta, Ga). WS1 was derived from FA19 as a spontaneous pyocin-resistant mutant deficient in either the synthesis or addition of heptose to the LOS core (29). JWS-1 was generated by transforming FA19 with chromosomal DNA from the terminally differentiated, nontransformable WS1 mutant and screening for loss of reactivity to the MAb 2-1-L8. Its LOS phenotype was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* DH5αMCR and JM101 were from Bethesda Research Laboratories (Bethesda, Md.). *S. typhimurium* LPS mutants, SA1377 (RfaC$^-$) (22), SL3600 (RfaD$^-$) (14), and SL1102 (RfaE$^-$) (33), were obtained from K. E. Sanderson of the University of Calgary *Salmonella* Genetic Stock Centre (Calgary, Canada). The plasmid pB37 containing *lsi-6* was kindly provided by E. S. Drazek (Department of Bacterial Disease, Walter Reed Army Institute of Research, Washington, D.C.).

All gonococcal strains were grown on GCMB agar or in GCMB broth (Difco Laboratories, Detroit, Mich.) supplemented with Kellogg's solution (32) and sodium bicarbonate (0.042%). *E. coli* and *S. typhimurium* strains were grown in L broth or on Luria-Bertani agar supplemented with kanamycin (Kan) (25 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (35 μg/ml) when appropriate (18).

The plasmid pKUP is a derivative of pK18 containing a gonococcal uptake sequence and has been described previously (23). Plasmid DNA was isolated by the alkaline-lysis method and subjected to $CsCl_2$ density centrifugation when appropriate (18). Curing experiments were performed by growing *S. typhimurium* transformants in L broth with SDS (0.005%) overnight. After several cycles of curing, individual colonies were screened for sensitivity to Kan (25 μg/ml).

Reagents and enzymes. All reagents were of molecular-biology grade or better. Acrylamide and bisacrylamide were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.); ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine were from Bio-Rad Laboratories (Richmond, Calif.); silver nitrate, formaldehyde, and ammonium hydroxide were obtained from Fisher Scientific Co. (Silver Spring, Md.); periodic acid was obtained from J. T. Baker, Inc. (Phillipsburg, N.J.); restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass.); and proteinase K was from Sigma Chemical Company (St. Louis, Mo.). MAb 2-1-L8 was generously provided by Wendell Zollinger, Walter Reed Army Institute of Research, Washington, D.C.

SDS-PAGE, transformations, and immunological techniques. Gonococcal

LOS and *S. typhimurium* LPS preparations were prepared from proteinase K-treated whole-cell lysates (12). Approximately 1-μg samples of LOS or LPS were run on 13% isocratic gels (23). *E. coli* transformations were performed with $CaCl_2$-competent cells (18). *S. typhimurium* transformations were performed by electroporation (Electro Cell Manipulator 600; BTX, San Diego, Calif.) following the manufacturer's recommended procedure. Gonococcal transformations were as follows: 10 μl of a JWS-1 overnight culture diluted in GCP–$MgCl_2$ (10 mM) to an approximate concentration of 10^4 CFU/ml was spotted on GCK plates, approximately 50 ng of the test DNA was added, and the plates were allowed to dry. After overnight growth, colonies were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) and screened for reactivity to the MAb 2-1-L8 (23).

Generation of FA19 chromosomal gene bank. Chromosomal DNA was isolated from strain FA19, the isogenic parent of JWS-1 (Fig. 1, step 1). FA19 chromosomal DNA was subjected to a partial, triple digestion with *Taq*$^{\alpha}$I, *Hin*PI, and *Hpa*II (Fig. 1, step 2). Fragments varying in size from 3 to 5 kb were isolated by sucrose density centrifugation and ligated into the *Acc*I site of pKUP (Fig. 1, steps 3 and 4). The gene bank was transformed into *E. coli* DH5αMCR, and Kan^r transformants were selected on LB media containing Kan and X-Gal (Fig. 1, step 5). Plasmid DNA was isolated from *E. coli* transformants in pools of 100 and used to transform JWS-1 (Fig. 1, step 6). JWS-1 transformants were tested for wild-type LOS expression by acquisition of reactivity to the MAb 2-1-L8 (Fig. 1, step 7). Individual *E. coli* transformants were screened from the pools capable of transforming JWS-1 to MAb 2-1-L8 reactivity, and individual clones containing the cloned DNA of interest were identified.

Plasmid deletion construction. Construction of unidirectional deletion mutants was performed with the Erase-A-Base system (Promega Corp., Madison, Wis.). Plasmid pJCL2 was digested with *Xma*I, and the ends were filled in with α-phosphorothioate nucleotides, digested with *Bam*HI, and treated with exonuclease III. At various time points samples were removed, treated with S1 nuclease, ligated, and used to transform *E. coli* DH5αMCR. Plasmid DNA was isolated from transformants and analyzed on agarose gels.

N. gonorrhoeae JCLΔ5 was constructed by introducing a 430-bp deletion within *rfaD* contained on pJCL2. This was done with the Erase-A-Base system (Promega Corp.) in a limited, bidirectional-erasing experiment. This deletion plasmid was subsequently used in allelic replacement experiments to introduce the *rfaD* deletion into the chromosome of *N. gonorrhoeae* FA19, as verified by PCR analysis, generating strain JCLΔ5.

DNA sequencing and analysis. DNA sequencing reactions of plasmid DNA were performed by the dideoxy method of Sanger et al. (25). When necessary, sequencing was performed with M13 single-stranded DNA (18). Reactions were performed with the Sequenase version II sequencing kit (United States Biochemicals, Cleveland, Ohio) and α-^{35}S-dATP (New England Nuclear, DuPont, Boston, Mass.). Sequencing products were run on a 4% acrylamide gel (55 cm by 0.2 mm; 7 M urea) with a 0.6-mm wedge in the last 10 cm. TBE (100 mM Tris, 83 mM boric acid, 1 mM EDTA) was used as the sequencing buffer. The DNA sequence was analyzed with GENEPRO version 4.2 (Riverside Scientific, Seattle, Wash.) and PCGene version 6.5 (IntelliGenetics, Mountain View, Calif.) software. Amino acid homology searches were conducted by using the GENINFO BLAST network service at the National Center for Biotechnology Information.

Reverse transcriptase PCR (RT-PCR). Total cellular RNA was isolated from *N. gonorrhoeae* FA19 and JCLΔ5 as follows. On ice, 1.5 ml of NaN_3 was added to 20 ml of cells that had been grown to mid-log phase. Cells were collected by centrifugation at 4,000 × *g* for 5 min, and the pellet was resuspended in 5 ml of lysis buffer (0.5% SDS, 0.02 M sodium acetate [pH 5.5]). Phenol (equilibrated with lysis buffer) was added (5 ml) and allowed to incubate at 65°C for 10 min. The mixture was centrifuged at 10,000 × *g*, and the phenol extraction was repeated on the supernatant. The supernatant was precipitated with 0.1 ml of NaCl (5 M) and 10 ml of 100% ethanol. Nucleic acid was collected by centrifugation at 14,000 × *g* for 20 min and washed with 75% ethanol. The pellet was resuspended in diethylpyrocarbonate-treated H_2O and subjected to DNase treatment following standard procedures. All labware was treated with RNaseZAP (Ambion Inc., Austin, Tex.) and all solutions were diethylpyrocarbonate treated when appropriate to minimize RNase contamination.

All PCR experiments were performed under the following reaction conditions: PCR, 94°C for 1 min, 52 to 62°C (depending on primer thermal denaturation) for 1 min, and 72°C for 1 min (30 cycles); RT-PCR, 48°C for 45 min, 94°C for 2 min, and 40 cycles at 94°C for 30 s, and 52 to 62°C (depending on primer thermal denaturation) for 30 s, and extension at 68°C for 2 min. PCR was performed with the GeneAmp PCR kit from Perkin-Elmer Cetus (Norwalk, Conn.), and the RT-PCR kit was from Promega Corp.

Primers used in PCR and RT-PCRs were obtained from the University of Maryland Protein and Nucleic Acids Facility and are as follows: internal *rfaE* fragment, 5′GCCCCAAGCCCATTCGGC (JL-2S) and 5′GCGCTCGACGCG CTGATGGT (JL-23); *pyrF* and *rfaE*, 5′TCGAAGTCTTAATGCGTAACCGG TGA (JL-7) and 5′GTCGTGGTTCATCGTATCGGAACACGC (JL-30); and *rfaE* and *rfaD*, 5′CGGACTACGGCAAAGGCGGTCTGT (JL-40) and 5′TGT GGAAAGTTCGGGATGGTCGAA (JL-41).

Nucleotide sequence accession number. The DNA sequence of *lsi-7* has been submitted to GenBank as an addition to a previously submitted sequence that has the accession number L07845.

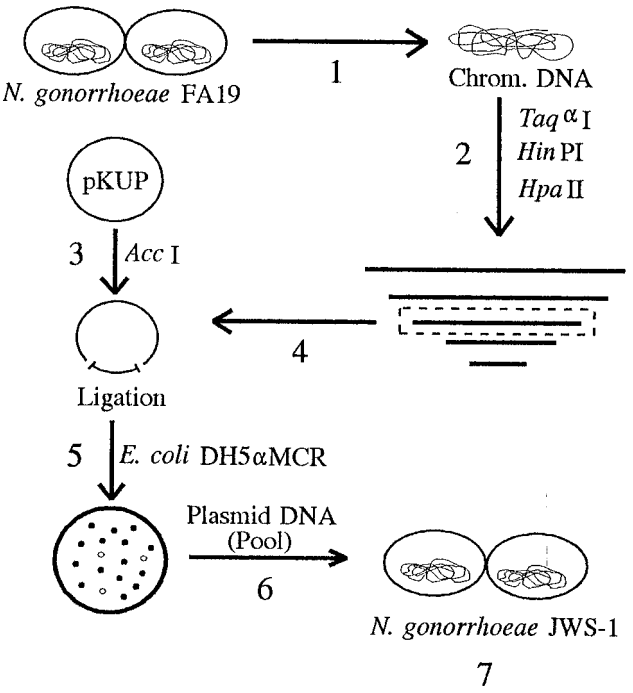

FIG. 1. Construction of an *N. gonorrhoeae* FA19 chromosomal gene bank and clone isolation. Numbers refer to specific steps outlined in Materials and Methods.

RESULTS

Isolation of a clone capable of restoring wild-type LOS expression to *N. gonorrhoeae* JWS-1. We screened a gene bank of FA19 DNA to isolate a DNA sequence capable of restoring wild-type LOS expression in JWS-1 (the construction scheme used is outlined in Fig. 1). Two *E. coli* transformants were identified and designated pJCL1 and pJCL2 and contained inserts of 4.0 and 3.5 kb, respectively. Restriction mapping indicated that the two clones overlapped and shared approximately 2.5 kb of DNA. Further analysis demonstrated that although pJCL1 was capable of repairing the genetic defect in JWS-1 by allelic replacement, it only contained part of the gene in which JWS-1 is deficient. pJCL2, which contained the complete gene, was chosen for further analysis.

LOS phenotype of *N. gonorrhoeae* JWS-1 and its complementation by pJCL2. The ability of pJCL2 to restore wild-type LOS expression to JWS-1 was initially based on MAb reactivity. The restoration was verified by SDS-PAGE of LOS isolated from MAb 2-1-L8-reactive transformants and compared with that of the parental strain, FA19. Figure 2 shows an SDS-PAGE gel of FA19 (the isogenic parent), WS1 (original, nontransformable heptoseless LOS mutant), JWS-1 (transformable derivative of WS1), and JWS-12 (JWS-1 transformed with pJCL2).

Strains WS1 and JWS-1 each produce a single, low-molecular-mass LOS band (lanes 2 and 3, respectively), while the parental strain, FA19, produces three visible bands (lane 1). The highest-molecular-mass band in lane 1, with an apparent molecular mass of 4,700-Da, binds the MAb 1B2, while the lowest-molecular-mass band, with an apparent molecular mass of 3,600-Da, binds the MAb 2-1-L8. The LOS of strain JWS-12 appears electrophoretically identical to that of the parental strain, FA19 (lane 4). Colony immunoblots on FA19 and JWS-12 show that both strains react with MAbs 1B2 and 2-1-L8 (data not shown). From these data we conclude that the

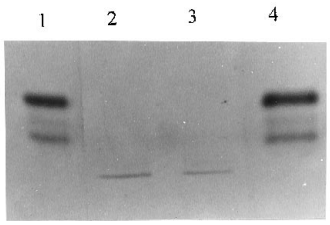

FIG. 2. SDS-PAGE analysis showing complementation of *N. gonorrhoeae* JWS-1. Gonococcal LOS was isolated as described in Materials and Methods and subjected to electrophoresis on a 13% polyacrylamide gel followed by silver staining. Lane 1, the parental strain FA19; lane 2, the heptoseless LOS mutant WS1; lane 3, JWS-1 (transformable derivative of WS1); lane 4, JWS-12 (JWS-1 transformed by pJCL2).

genetic defect in WS1 was successfully introduced into JWS-1 and that pJCL2 is capable of complementing the genetic defect in JWS-1.

Location of the gene on pJCL2. To determine the region of the insert capable of restoring JWS-1 to wild-type LOS expression, sequential unidirectional deletions were constructed. Figure 3 lists a subset of pJCL2 deletion clones analyzed and their relative sizes. These clones were used to transform JWS-1 by allelic replacement, and the loss of transformability to wild-type LOS expression was monitored by MAb 2-1-L8 reactivity. Since the vector contains a gonococcal uptake sequence, the loss of transformability would only result from the deletion of the transforming locus. Since pB37 is unable to transform JWS-1 to MAb 2-1-L8 reactivity but pJCL9 and pJCL1 are able to, the mutation in JWS-1 is most likely at the 3′ end of *lsi-7* (*rfaE*).

Characterization of the insert contained in pJCL2. The DNA sequence analysis identified an open reading frame

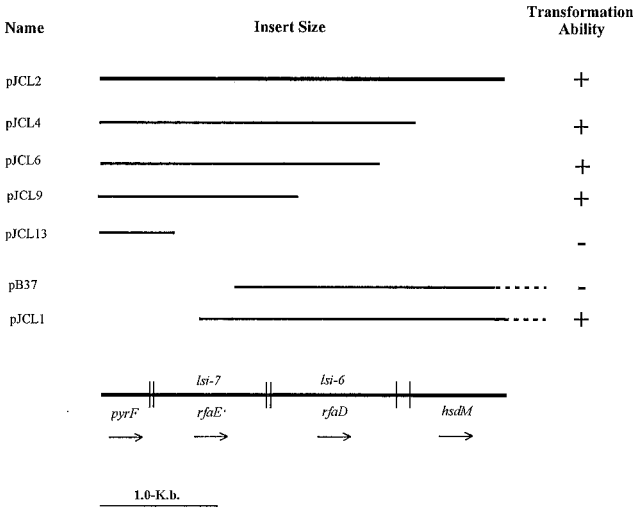

FIG. 3. Deletion mapping and ORF analysis of pJCL2. The leftmost column lists the deletion constructs, the middle column shows the relative sizes of the deletion constructs and their relative locations with respect to the parental plasmid, pJCL2, and the rightmost column indicates the transformability (ability [+] or inability [−] to be transformed) of the deletion construct as monitored by loss of reactivity to the MAb 2-1-L8. The locations of ORFs contained on pJCL2 and their identities based on sequence analysis (for *hsdM* and *pyrF*) or based on sequence and *S. typhimurium* LPS complementation studies (for *rfaD* and *rfaE*) are shown at the bottom. The direction of transcription for each locus is indicated by an arrow. The loci labeled *lsi-6* and *lsi-7* reflect the initial identification of these loci as being involved in LOS biosynthesis prior to the determination of their exact function.

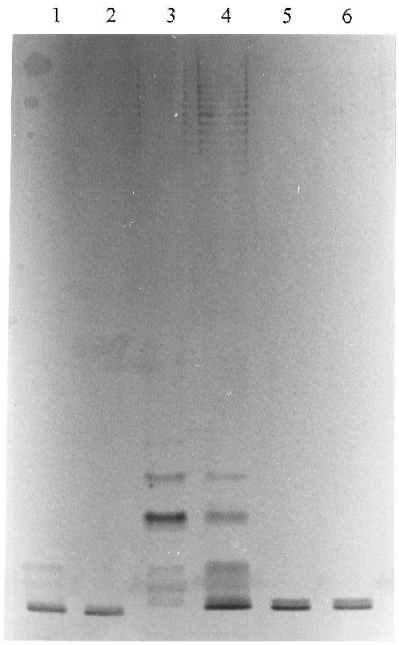

FIG. 4. SDS-PAGE analysis demonstrating complementation in *trans* of *S. typhimurium* LPS mutants on a 13% polyacrylamide gel that has been silver stained. Lane 1, *rfaD* mutant; lane 2, *rfaE* mutant; lane 3, *rfaD* (pJCL2); lane 4, *rfaE* (pJCL2); lane 5, *rfaE* transformant (cured of pJCL2); lane 6, *rfaE* (pKUP).

(ORF) designated *lsi-7* (for lipooligosaccharide involved). *lsi-7* consists of 963 bp and codes for a predicted protein of 320 amino acids with a molecular weight of 34,699. Immediately downstream (4 bases) of the *lsi-7* coding region is a gonococcal uptake sequence. Protein homology searches of the predicted *lsi-7* translation product identified significant homology between this region and several enzymes involved in the phosphorylation of sugars.

Additional sequence analysis identified a partial ORF located 5′ to *lsi-7*. Although the DNA sequence of this ORF is incomplete, its deduced translation product has significant homology to *pyrF* of *P. aeruginosa*, a gene involved in nucleotide biosynthesis. On the 3′ end adjacent to *lsi-7* an additional ORF was identified. This ORF consists of 1,002 bp and has previously been identified as an LOS biosynthetic gene and designated *lsi-6* (6). *lsi-6* has 78% amino acid identity to the *rfaD* gene of *S. typhimurium* and has subsequently been designated *rfaD* in the gonococcus. On the far 3′ end of pJCL2, a partial ORF whose predicted translation product has homology to *E. coli hsdM*, a type I methylase gene, was identified. The relative sizes and locations of all ORFs are shown at the bottom of Fig. 3. All ORFs are translated in the same direction, from left to right, as shown in Fig. 3.

Complementation of *S. typhimurium* LPS rough mutants. Since previous studies had demonstrated that LOS biosynthetic genes are capable of complementing enteric LPS mutants (24, 34), we wished to determine whether pJCL2 was able to complement LPS mutants deficient in the synthesis or addition of heptose to the LPS core of *S. typhimurium*. It is postulated that the synthesis and addition of heptose to enteric LPS requires five gene products (4, 7, 26, 30). The ability of pJCL2 to complement, in *trans*, *S. typhimurium* LPS mutants was tested on the following mutants: the transferase (*rfaC*), the epimerase (*rfaD*), and the synthase (*rfaE*). The data contained in Fig. 4 demonstrate complementation of *S. typhimurium rfaD*

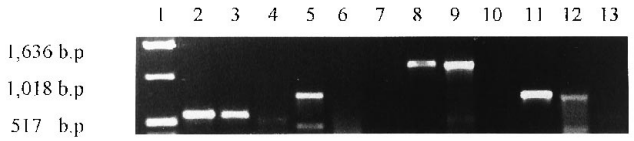

FIG. 5. RT-PCR and PCR analyses of RNA transcripts and chromosomal DNA from *N. gonorrhoeae* FA19 and JCLΔ5. Lanes 2, 5, 8, and 11 are the results of PCRs using chromosomal DNA as the template, and lanes 3, 6, 9, and 12 are from RT-PCRs using total cellular RNA as the template. Lanes 4, 7, 10, and 13 are control reactions in which RNase has been added to the RT-PCR mixture to demonstrate that any amplification is RNA specific. Lane 1, molecular weight markers; lanes 2 and 3, primers specific for an internal fragment of *rfaE* (from FA19; expected fragment, 577 bp); lanes 5 and 6, primers specific for a fragment containing part of *pyrF* and *rfaE* (from FA19; expected fragment, 781 bp); lanes 8 and 9, primers designed to amplify a fragment containing part of *rfaD* and *rfaE* (from FA19; expected fragment, 1,191 bp); lanes 11 and 12, same primers as used in lanes 8 and 9 but with DNA or RNA from strain JCLΔ5 used as the template (expected fragment, 761 bp).

and *rfaE* by pJCL2 in *trans*.

Since the *rfaD* and *rfaE* mutants are defective in heptose biosynthesis, their LPS is predominantly of low molecular weight (rough) as visualized by SDS-PAGE (Fig. 4, lanes 1 and 2, respectively). The *rfaD* mutant produces a small amount of a larger LPS structure due to the limited incorporation of the wrong heptose epimer into its LPS (lane 1) (14). Complementation by pJCL2 results in the production of a higher-molecular-weight LPS, characterized by a typical LPS ladder in the complemented *rfaD* and *rfaE* mutants (lanes 3 and 4, respectively). The *rfaE* mutant complemented by pJCL2 still produces some low-molecular-weight components characteristic of a rough LPS mutant (lane 4) due to the instability of the plasmid in this strain (unpublished observations). The result of this instability is a mixed population containing, in part, transformants which have deleted all, or part, of the insert in pJCL2.

SDS curing of pJCL2 from an *rfaE* transformant resulted in the complete restoration of the rough LPS phenotype, verifying the complementation of the mutant in *trans* (lane 5). As an additional control, the vector base of pJCL2, pKUP, was used to transform *rfaE*. As lane 6 shows, pKUP had no effect on LPS synthesis in this mutant. An *rfaC* mutant deficient in heptose addition to the LPS core was unable to be complemented by pJCL2 (data not shown). On the basis of these data we propose that *lsi-7* be given the gene designation *rfaE* in gonococci.

RT-PCR analysis of *rfaD* and *rfaE* transcripts in JWS-1. To determine if *rfaD* and *rfaE* are transcriptionally linked, RT-PCRs were performed. Figure 5 shows an ethidium bromide-stained agarose gel of PCR and RT-PCR products. Lane 2 shows the PCR product obtained when an internal *rfaE* fragment of 577 bp is amplified from strain FA19. Lane 3 consists of the same reaction performed on FA19 RNA by RT-PCR. Lane 4 is a control reaction in which RNase has been added to the RT-PCR mixture. Together these lanes demonstrate the presence, and successful amplification, of the *rfaE* gene from an RNA template. Lanes 5, 6, and 7 are a similar reaction series using primers designed to amplify a 781-bp fragment containing part of the *pyrF* and *rfaE* genes. The absence of an amplification product in Lane 6 indicates that these genes are not part of the same transcriptional unit. Lanes 8, 9, and 10 use primers designed to amplify a 1,191-bp fragment containing part of the *rfaD* and *rfaE* genes. The presence of a 1,191-bp product in lane 9 indicates that the two genes are present on the same RNA transcript. In order to verify that the amplification product seen in lanes 8 and 9 represents the genes of interest, the same reaction series was repeated with strain JCLΔ5 (lanes 11, 12, and 13). This strain contains a partial deletion in *rfaD* and should generate a 761-bp PCR product.

DISCUSSION

In order for *N. gonorrhoeae* to make an LOS molecule, a cell must express the needed sugar transferases and possess the ability to make the needed biosynthetic intermediates. Synthesis of the gonococcal core structure requires the synthesis of heptose and its addition to 2-keto-3-deoxyoctulosonic acid. The gonococcal pyocin mutant JWS-1 is unable to incorporate heptose into its core structure; this indicates a deficiency in heptose metabolism or in the ability to add heptose to 2-keto-3-deoxyoctulosonic acid. In LPS synthesis in *E. coli* and *S. typhimurium*, five gene products are postulated to be involved in heptose synthesis and addition to 2-keto-3-deoxyoctulosonic acid: an isomerase, a mutase, a synthase (RfaE), an epimerase (RfaD), and a transferase (RfaC). We postulated that JWS-1 is deficient in one of these five gene products and devised a strategy to clone a locus capable of complementing this defect.

We used a deletion analysis-allelic replacement strategy to localize the region on pJCL2 that was able to complement the defect found in JWS-1. DNA sequence analysis of this region identified a single ORF, designated *lsi-7* (*rfaE*). When the predicted translation product of this ORF was used to probe protein databases, significant homology between Lsi-7 and proteins involved in the phosphorylation of sugars was found. DNA sequence analysis identified another gene involved in heptose biosynthesis, *lsi-6* (*rfaD*). Further analysis indicated that 5′ to *rfaE* was an ORF with significant homology to *pyrF* of *P. aeruginosa* and 3′ of *rfaD* was an ORF with significant homology to the *hsdM* gene of *E. coli*. Since sequence analysis indicates that *rfaD* and *rfaE* are translated in the same direction and there are no obvious transcriptional termination sequences between them, it suggested that they were part of the same transcriptional unit. Sequence analysis also identified a potential transcriptional promoter immediately upstream of *rfaE*, suggesting that *rfaE* is the first gene transcribed.

In *E. coli* and *S. typhimurium*, the *rfaD* and *rfaE* genes are separated by several minutes on the chromosome (26). Since these two genes were genetically linked in the gonococcus, we wished to determine if they were also transcriptionally linked. We used RT-PCR analysis to demonstrate that *rfaD* and *rfaE* are part of the same transcriptional unit in the gonococcus (Fig. 5). Using this procedure, we were unable to demonstrate transcriptional linkage of *rfaE* with *pyrF*. Therefore, we concluded that these genes, although positionally linked, are on different transcripts.

To verify that the pJCL2 contained the gonococcal homologs of *rfaD* and *rfaE*, we employed complementation analysis using *S. typhimurium* LPS mutants. Complementation studies in *trans*, verified via SDS-PAGE analysis, indicated that pJCL2 contains functional *rfaD* and *rfaE* homologs. These data, in conjunction with deletion analysis, indicate that JWS-1 is deficient in LOS biosynthesis because of a genetic lesion in the gonococcal homolog of *rfaE*, the gene that encodes ADP-heptose synthase.

Although certain analogous components between enteric LPS and gonococcal LOS exist, chromosomal arrangements of the biosynthetic genes themselves differ widely. The majority of the genes involved in enteric LPS core biosynthesis occur in two clusters of linked genes (26). Only two of the at least four gene products thought to be involved in heptose biosynthesis have been identified. These genes, *rfaE* and *rfaD*, are not part of the same transcriptional unit in *S. typhimurium* (26). What is emerging with respect to biosynthesis in the gonococcus is the

presence of several clusters of genes involved in LOS biosynthesis. The operon described by Gotschlich (9) is involved in the biosynthesis of the α-oligosaccharide and consists of five glycosyl transferases. We have identified another operon involved in the synthesis of the β- and γ-chains (30a). The study presented in this work represents a third group of linked LOS biosynthetic genes (*rfaD* and *rfaE*).

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