Genetic Evidence that *Neisseria gonorrhoeae* Produces Specific Receptors for Transferrin and Lactoferrin

KEVIN J. BLANTON,1+ GOUR D. BISWAS,1 JOYCE TSAL,1 JOHN ADAMS,2‡ DAVID W. DYER,3§ SONIA M. DAVIS,3 GARY G. KOCH,3 PRANAB K. SEN,3 AND P. FREDERICK SPARLING1,2*  

Departments of Microbiology and Immunology1 and Medicine,2 School of Medicine, and Department of Biostatistics, School of Public Health,3 University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Received 2 August 1989/Accepted 22 June 1990

Transferrin (TF) and lactoferrin (LF) are probably the major sources of iron (Fe) for *Neisseria gonorrhoeae* in vivo. We isolated mutants of *N. gonorrhoeae* FA19 that were unable to grow with Fe bound to either TF (TF") or LF (LF") or to both TF and LF ([TF LF]). The amount of Fe internalized by each of the mutants was reduced to background levels from the relevant iron source(s). The wild-type parent strain exhibited saturable specific binding of TF and LF; receptor activity was induced by Fe starvation. The TF"-specific or LF"-specific mutants were almost completely lacking in receptor activity for TF or LF, respectively, whereas the [TF LF]" mutants bound both TF and LF as well as the wild-type strain. All mutants utilized citrate and heme normally as Fe sources. These results demonstrate that ability to bind TF or LF is essential for gonococci to scavenge appreciable amounts of Fe from these sources in vitro. In addition, the TF and LF Fe acquisition pathways are linked by the mutual use of a nonreceptor gene product that is essential to Fe scavenging from both of these sources; this gene product is not required for Fe acquisition from other sources.

Iron (Fe) is an essential nutrient for the growth of almost all microorganisms. Most bacteria produce siderophores (low-molecular-weight iron chelators) to compete with existing Fe-binding compounds, or to solubilize Fe, which forms highly insoluble complexes in aerobic aqueous environments (18–20). These bacteria subsequently acquire the siderophore-bound Fe through a receptor-mediated pathway. Most siderophore-producing bacteria are able to exist both in inanimate environments in which all Fe is in the form of insoluble complexes and in environments in which Fe is bound to soluble Fe chelators. *Neisseria gonorrhoeae* is relatively unusual in its apparent absence of siderophore production (22, 36, 37) and in that it is found naturally only in humans, an environment in which Fe is sequestered by a variety of soluble Fe-carrying glycoproteins or heme compounds. Perhaps for these reasons gonococci have evolved mechanisms for obtaining Fe which are quite different from those utilized by siderophore-producing bacteria.

The Fe sources available to gonococci vary depending on the site of infection. Lactoferrin (LF) is probably the major Fe source during localized infections on the mucosal surfaces of the urogenital tract, rectum, pharynx, and eye (3, 13), whereas transferrin (TF) is probably the major Fe source during invasive infections of the bloodstream, synovial fluid, and cerebrospinal fluid (35). All gonococcal strains utilize TF as an Fe source (16) and most gonococcal strains are able to utilize LF, heme, hemin, hemoglobin, and certain hydroxamate siderophores produced by other bacteria (15, 16, 36, 38). However, because sources other than TF and LF either are utilized inefficiently or are not readily available to the gonococcus during an infection (7, 16, 17, 36, 37), they are believed to be minor Fe sources.

The mechanisms used by gonococci to scavenge Fe from TF or LF are not completely understood. Both TF and LF are only partially Fe saturated in vivo and bind Fe with high affinity, yet gonococci are able to remove Fe very efficiently from these proteins. The mechanisms are energy requiring, and full expression of the Fe uptake system requires prior growth under Fe starvation conditions (14). The ability to acquire Fe from TF and LF saturates at about 1 μM for both proteins and requires direct contact between the protein and the gonococcal cell surface (14), suggesting that gonococci may possess saturable receptors for both TF and LF. The removal of Fe from TF or LF does not appear to involve internalization of the receptor-ligand complex (14).

In these respects, the gonococcus closely resembles the meningococcus, another non-siderophore-producing organism (1, 36). Saturable and specific receptors for both TF and LF were described recently in the meningococcus (26, 27, 33), and the TF receptor was shown to be important to Fe scavenging (33). The biochemical identities of the meningococcal TF and LF receptors and the functional importance of the LF receptor remain unclear (26–28, 33). Similar receptors have been suggested for the gonococcus (11); however, the existence of a gonococcal TF or LF receptor has not been definitively demonstrated or characterized. Receptors for binding TF or LF have also been suggested for *Haemophilus influenzae*, *Trichomonas vaginalis*, *Bordetella pertussis*, and *Mycoplasma pneumoniae* (23–25, 32), each of which is found naturally only in humans and none of which is known to produce siderophores.

In this communication we provide genetic and biochemical evidence for specific gonococcal receptors for both TF and LF, and we demonstrate the importance of these receptors to Fe scavenging.

* Corresponding author.
† Present address: Yale University School of Medicine, New Haven, CT 06510.
‡ Present address: Knoxville Infectious Disease Consult, Knoxville, TN 37916.
§ Present address: Department of Microbiology, State University of New York, Buffalo, NY 14214.
**TABLE 1. N. gonorrhoeae strains used in this study**

| Strain | Relevant genotype<sup>a</sup> | Relevant phenotype | Source or method of construction |
|--------|-------------------------------|-------------------|----------------------------------|
| FA19   | Prototroph                    | LF<sup>+</sup> TF<sup>+</sup> | Clinical isolate (15)           |
| FA248  | *trf-2*                       | LF<sup>+</sup>    | F62 derivative (2)              |
| FA1012 | *trf-3*                       | LF<sup>+</sup>    | F29 derivative (1)              |
| FA1092 | *trf-5*                       | LF<sup>+</sup>    | Clinical isolate                |
| FA1095 | *trf-6*                       | LF<sup>+</sup>    | Clinical isolate                |
| FA3000 | *trf-4*                       | LF<sup>+</sup>    | Clinical isolate                |
| FA3002 | *trf-7*                       | LF<sup>+</sup>    | Clinical isolate                |
| FA6246 | *trf-2*                       | LF<sup>+</sup>Rif<sup>a</sup> | A transformant of FA248         |
|        | Str<sup>a</sup>               |                   |                                  |
| FA6303 | tlu-1                         | [LF TF]<sup>-</sup> | tlu-1 mutation → FA19<sup>9</sup> |
| FA6338 | *trf-1*                       | TF<sup>-</sup>     | trf-1 mutation → FA19<sup>9</sup> |
| FA6342 | *trf-3*                       | TF<sup>-</sup>     | trf-3 mutation → FA19<sup>9</sup> |
| FA6353 | *trf-1*                       | LF                | trf-1 mutation → FA19<sup>9</sup> |
| FA6355 | tlu-1 *trf-1*                 | LF<sup>-</sup>, TF<sup>-</sup> | trf-1 mutation → FA6353<sup>9</sup> |
| FA6356 | *trf-2*                       | TF<sup>-</sup>     | trf-2 mutation → FA19<sup>9</sup> |
| FA6366 | *trf-5*                       | TF<sup>-</sup>     | trf-5 mutation → FA19<sup>9</sup> |
| FA6367 | *trf-4*                       | TF<sup>-</sup>     | trf-4 mutation → FA19<sup>9</sup> |
| FA6370 | tlu-2                         | [LF TF]<sup>-</sup> | tlu-2 mutation → FA19<sup>9</sup> |
| FA6378 | tlu-3                         | [LF TF]           | tlu-3 mutation → FA19<sup>9</sup> |
| FA6381 | tlu-4                         | [LF TF]           | tlu-4 mutation → FA19<sup>9</sup> |
| FA6382 | tlu-5                         | [LF TF]<sup>-</sup> | tlu-5 mutation → FA19<sup>9</sup> |
| FA6411 | *trf-1* *trf-2*               | LF<sup>-</sup>TF<sup>-</sup> | trf-1 mutation → FA248<sup>9</sup> |

<sup>a</sup> *trf*, LF receptor function; *trf*, TF receptor function; tlu, TF, LF utilization functions.

<sup>b</sup> Ethyl methanesulfonate mutants of FA19 were isolated by streptomycin enrichment. The mutations were then transferred back into FA19 by transformation, selecting for a Rif<sup>+</sup> donor marker and scoring for inability to use either LF or TF, as described in Materials and Methods.

<sup>c</sup> DNA isolated from Str<sup>a</sup> derivative of FA6338.

---

**MATERIALS AND METHODS**

**Bacterial strains and media.** All strains used in this study are described in Table 1. Gonococci were routinely maintained on GCB agar (Difco Laboratories, Detroit, Mich.), containing 1% Kelloggs supplement I and 0.1% Kelloggs supplement II (9). Cultures were incubated at 37°C with a 5% CO₂ atmosphere. Fe uptake experiments and TF- or LF-binding experiments utilized gonococcal chemically defined Fe-depleted medium (CDM) described previously (36). The concentration of Fe remaining in Fe-depleted CDM was ≤0.1 µM as determined by atomic absorption spectroscopy. Glassware was acid washed to remove Fe, as described previously (16). Growth in CDM broth was monitored by optical density, using a Klett-Sumner colorimeter with a green filter. CDM-agar plates contained 1 to 1.5% sterile agar or 0.75% sterile agarose (Bethesda Research Laboratories, Gaithersburg, Md.) and 0.5 to 1% sterile potato starch (Sigma Chemical Co., St. Louis, Mo.). Agarose or purified agar was prepared by the procedure described by Catlin (4). CDM-O agar plates or broth were not supplemented with Fe, but agar plates contained approximately 2 µM Fe due to Fe present in the agar (16). CDM + Fe plates and broth were supplemented with 50 µM Fe(NO₃)₃. The CDM + TF and CDM + LF plates were supplemented with 2 to 5 µM apo-TF or apo-LF, respectively, which resulted in approximately 20 to 50% Fe saturation of TF or LF due to the residual Fe present in the agar. For designated experiments, the CDM also contained 25 µM desferoxamine mesylate (Desferal; Ciba Pharmaceutical Co., Summit, N.J.) to chelate any remaining free Fe.

**Fe sources.** Human TF (Sigma) was at least 98% pure and significantly Fe-free according to the supplier and as verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was relatively Fe-free. Human LF (a gift of S. V. Pizzo, Duke University Medical Center) was approximately 95% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was relatively Fe-free. Human LF was also obtained from U.S. Biochemical Corp., Cleveland, Ohio, and was >98% pure according to the supplier. For the Fe uptake experiments, TF and LF were Fe loaded with ⁵⁵FeCl₃ (New England Nuclear, Boston, Mass.) as described previously (14); this resulted in 15 and 27% Fe saturation of TF and LF, respectively, as determined by the Ferrozine assay for Fe (31). Ferric citrate complexes were prepared by adding a 10-fold molar excess of sodium citrate to ⁵⁵FeCl₃. For the liquid phase binding experiments, TF was 100% saturated with Fe(NO₃)₃, and was labeled with [¹²⁵I]NaI (Amersham Corp., Arlington Heights, Ill.). The quality of the [¹²⁵I]TF was verified both by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by its ability to donate Fe normally to growing gonococci, using methods described previously (33).

**Mutagenesis and isolation of mutants.** The procedure for isolating Fe uptake mutants was a modification of one used previously to isolate similar mutants of N. meningitidis (5). Nonpiliated gonococci were scraped from overnight growth on CDM + Fe plates, suspended in CDM + Fe broth, and grown into log phase, when they were diluted back to 10⁶ CFU/ml in CDM-O. Ethyl methanesulfonate (Sigma) was added to a concentration of 1%, and the cells were incubated for 20 to 30 minutes with shaking, resulting in an 80 to 95% decrease in viability as compared with untreated cells. Following incubation, the cells were washed three times by centrifugation at room temperature and suspended in an equal volume of CDM plus 5 µM Fe(NO₃)₃. The mutagenized cells were incubated overnight at 37°C in a still culture, washed once, suspended into CDM containing 5 µM 25% Fe-saturated TF or LF, and incubated at 37°C in a 5% CO₂ shaking incubator for 4 to 5 h. Streptomycin (M. Suffness, National Cancer Institute, Bethesda, Md.) was added to the culture at 0.2- to 0.6-µg/ml final concentration, and the culture was incubated at 37°C in a 5% CO₂ shaking incubator for 2 h, which resulted in 0.01 to 0.0001% survivorship. The cells were then washed three times by centrifugation, suspended in fresh CDM, and plated onto GCB agar plates. Survivors were scored for ability to grow with TF, LF, or Fe(NO₃)₃ by transferring the colonies to CDM agar plates containing one of these Fe sources. Mutants unable to use either TF or LF were tested for ability to grow on CDM plates containing heme, hemoglobin, or Fe citrate.

**Genetic procedures.** To construct an isogenic set of mutants, mutations were transformed back into FA19 by conjugation, using the procedure described previously (2). Transformation crosses were then performed between these transformants to determine relative linkage between various mutations. Spontaneous drug-resistant mutants were isolated for representative Fe uptake mutants on GCB agar plates containing 5 µg of rifampin (Rif), 5 µg of naladixic acid (Nal), or 100 µg of streptomycin (Str) per ml; these Rif<sup>R</sup>, Nal<sup>R</sup>, or Str<sup>R</sup> derivatives of the Fe uptake mutants were used to prepare transforming DNA. Frequencies of transformation for the unlinked drug resistance marker served as an internal control for the quality of the donor DNA. To control for differences in competency, recipients were transformed with FA19 DNA. This allowed calculation of a recombination index (RI) (8, 10). For example, the RI in a transformation cross between two TF<sup>+</sup> mutants = (TF<sup>+</sup> transformants using mutant DNA/drug<sup>-</sup> transformants using mutant DNA) × (TF<sup>-</sup> transformants using wild-type DNA/drug<sup>-</sup> transformants using wild-type DNA).
DNA)\times (drug' transforms using FA19 DNA/TF\^+ transforms using FA19 DNA).

Transforming DNA was isolated by suspending gonococci in 10 M Tris–1 mM EDTA (TE), pH 8.0, and adding of sodium sarcosinate to a final concentration of 0.5% to effect cell lysis. This crude DNA was diluted 1:500 in TE to give a final concentration of sodium sarcosinate of 0.001%; this concentration did not affect the viability of the competent cells (29). In certain experiments, transforming DNA was isolated by the procedure described by Marmur (12). Competent cells were obtained by growing pilated gonococci in CDM-O broth until late log phase and diluting the cells to 10\^5 CFU/ml. Transformation procedures followed the protocols described previously (2). TF\^+ or LF\^+ transforms were selected on CDM agar plates supplemented with Desferal and 25% saturated 2.5 \\mu M TF or LF, respectively. Drug-resistant transforms were selected on GCB agar plates overlaid with the appropriate antibiotic after allowing 5 h for phenotypic expression of the introduced marker (30).

57Fe uptake. The Fe uptake protocol was adapted from methods described recently (5, 14). Cells were grown overnight on CDM+Fe plates, suspended in CDM-O broth at an optical density of 15 to 20 Klett units, and incubated with shaking at 37°C in a 5\% CO\textsubscript{2} atmosphere to induce Fe starvation. Fe-starved cells were harvested during late exponential phase and diluted 1:1 in fresh CDM-O or CDM-O containing 40 mM potassium cyanide (KCN). During a 15-min incubation at 37°C, viable counts were determined. Cells were then diluted 1:1 in CDM containing partially 57Fe-saturated 5 \\mu M TF, LF, or citrate. Duplicate samples were removed after 10 and 30 min of incubation at 37°C, and 57Fe uptake was determined (14).

Equilibrium TF-binding assay. Specific binding of TF to gonococci used a protocol similar to that described for the meningococcus (33). Briefly, iron-replete cells taken from overnight growth on GCB agar plates were suspended in CDM-O broth to an optical density of 20 Klett units and were incubated at 37°C in 5\% CO\textsubscript{2} with shaking to induce Fe starvation. After 3.5 mass doublings (with back-dilution after the second doubling), cells were pelleted and suspended in a 1/50 volume of CDM plus 1% bovine serum albumin. The suspensions contained 0, 0.25, 0.5, 1.0, 2.0, or 5.0 \mu M \textsuperscript{125}I-TF in the presence and absence of a 100-fold excess of cold TF. After 20 min of incubation at 25°C, the cells were washed twice with pelleting and suspending in equal volumes of CDM-O plus 1% bovine serum albumin. Cell-associated radioactivity was determined in a Packard Minosx gamma (\gamma) Auto 5000 series counter. Specific binding was calculated as the amount of \textsuperscript{125}I-TF bound minus the amount of \textsuperscript{125}I-TF bound in the presence of a 100-fold excess of cold TF.

Dot blot TF- and LF-binding assay, using ligand dilutions. Cells were grown overnight on CDM-O plates and suspended in CDM-O broth. Approximately 10\^5 CFU were applied to each well of one row of a Miniblot apparatus (Millipore Corp., W. Bedford, Mass.) and were filtered onto nitrocellulose; different strains were applied to separate rows. The filter was dried, blocked in blotto (0.5\% skim milk, 50 mM Tris [pH 7.5], 150 mM NaCl), and cut into vertical strips. The nitrocellulose strips were incubated in blotto containing serial dilutions of TF- or LF-horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch, Westgrove, Pa.). The remainder of the procedure was as described by Schryvers and Morris (27), with the following modifications: during the ligand-binding step, cells were incubated at 25°C (instead of 37°C), and the blocking solution for the LF-HRP contained 500 mM NaCl (instead of 150 mM).

Dot blot TF-binding assay, using cell dilutions. Cells were grown overnight on CDM-O and CDM+Fe plates. The cells were suspended in CDM-O, and a suspension of approximately 10\^8 CFU was applied to the first column of the Miniblot apparatus, one strain per row. Each successive column contained a 1:1 dilution of the cells in the previous column. The cells were filtered onto the nitrocellulose. The filters were dried, blocked as above, and incubated with 500 ng of TF-HRP per ml. The dot blots were developed as above. This assay allowed a more sensitive analysis of ligand binding to the Fe-sufficient cells (with reduced receptor expression) and to the TF receptor mutants (with apparent reduction in receptor affinity).

RESULTS

Isolation of TF and LF utilization mutants. By use of ethyl methanesulfonate mutagenesis followed by streptomycin enrichment (5), we isolated mutants of FA19 (TF\^+ LF\^+), that were unable to grow with TF or LF or both as Fe source. Mutants occurred at a frequency of 0.1 to 1\% among survivors of streptomycin enrichment. In eight experiments, a total of 105 Fe utilization mutants were isolated. These mutants fell into three phenotypic classes: 55 were unable to use TF, but could use LF (TF\^-); 2 were unable to use LF, but could use TF (LF\^-); and 48 were unable to use either TF or LF ( TF LF\^-). Each of the tested mutants (FA6303, FA6338, FA6342, FA6353, FA6356, FA6366, and FA6367) grew normally on CDM plates supplemented with heme, hemoglobin, or Fe-citrate. Each tested mutant was relatively stable, reverting to TF\^+, LF\^+, or [TF LF\^-] at a frequency of 10\^-6 or less. Representative mutations from each of the three phenotypic classes were transformed back into the parental strain FA19 by conjugation to help remove unlinked, unrelated mutations resulting from the chemical mutagenesis. Most of the mutants we discuss are the result of back-transformations; all strains used in this study are described in Table 1.

Mapping of the mutational sites. Loci for the TF\^+, LF\^-, and [TF LF\^-] phenotypes were designated trf (TF receptor function), lrf (LF receptor function), and tluf (LF, LF utilization), respectively. Transformation experiments were performed to compare relative linkage between representative trf, lrf, and tluf mutations. Since transformation is intrinsically variable, the linkage relationships were expressed as an RI; an RI of <1.0 generally denotes linkage between markers (8). Analysis-of-variance methods were used to determine whether RIs were significantly <1.0. Statistical analysis was complex, and details are included in the Appendix. RIs calculated from an analysis of variance that included components for recipient and donor are displayed in Table 2 with corresponding observed ratios. The recipient strain (RIP) in initial recombination experiments was FA6355, a constructed double mutant (trf\^- lrf\^-). Donor DNA (RIP\_) was obtained from wild-type strain FA19 as well as mutant strains FA1012 ( lrf\^-), FA6342 (trf\^-), and FA6378 (tluf\^-). Transformation of FA6355 with donor DNA from wild-type strain FA19 produced almost equal numbers of TF\^+ and LF\^+ transforms (Table 2). However, donor DNA from FA6342 (trf\^-) produced many fewer TF\^+ transforms, and donor DNA from FA1012 (lrf\^-) produced many fewer LF\^+ transforms, as compared with other selected markers and as compared with FA19. These results indicated linkage of trf\^- to trf\^- and lrf\^- to lrf\^-3. Next, with FA6355
TABLE 2. Transformation between mutants unable to use TF, LF, or TF and LF

| DNA | Transforms per ml | LF⁺ | TF⁺ | RI⁺ | LF⁺ | TF⁺ |
|-----|------------------|-----|-----|-----|-----|-----|
|     |                   |     |     |     |     |     |
| FA19 | 5.9 × 10⁴ | 5.6 × 10⁴ | 3.4 × 10⁴ | 1.0 | 1.0 | 1.0 |
| FA1012 lrfl-3 | 2.8 × 10⁴ | 7.1 × 10⁴ | 3.0 × 10⁴ | 0.05 | 0.06 | 1.44 |
| FA6342 trfl-3 | 1.5 × 10⁵ | 1.1 × 10⁴ | 5.0 × 10⁴ | 1.73 | 1.28 | 0.13 |
| FA6378 tlu-3 | 1.6 × 10⁵ | 1.2 × 10⁵ | 5.0 × 10⁴ | 1.85 | 1.34 | 1.46 |

* Recipient strain, FA6355 lrfl-1 trfl-1.
* RIs of <1.0 suggest linkage of markers, whereas RIs of ≥1.0 suggest nonlinkage of markers; see text for explanation.
* Values for wild-type FA19 DNA were normalized to 1.0, for purpose of comparison to the other strains. The RIs for FA1012, FA6342, and FA6378 are the ratio of the observed RI to the RI for FA19. Values are the means of two experiments.
* Estimated RI values were calculated based on analysis of variance. 95% confidence intervals are given in parentheses.

(trfl-1 lrfl-1) as a recipient strain, donor DNA from FA6342 (trfl-3) and FA1012 (lrfl-3) produced as many LF⁺ and TF⁺ transformants, respectively (RIs, >1.0), as did the parental strain FA19. This suggested that the trfl-3 and lrfl-3 mutational sites were unlinked. Donor DNA from FA6378 (tlu-3) produced as many TF⁺ and LF⁺ transformants of FA6355 (trfl-1 lrfl-1) as did the parental strain FA19. This indicated that the tlu-3 mutation that produced the [TF LF⁻] phenotype was unlinked to either trfl-1 or lrfl-1. These conclusions were verified with analysis-of-variance methods.

**TF⁺ mutants are linked.** DNA from crude lysates of 33 originally isolated TF⁺ mutants were used to transform a competent TF⁻ mutant, FA6338 (trfl-1). In all cases the RIs were <0.5 (data not shown), suggesting that the mutational sites of all TF⁺ mutants were linked genetically.

TF⁻ strains FA6338, FA6342, FA6356, FA6366, and FA6367 were used in reciprocal transformations, with each strain being both a DNA donor and a recipient for each of the other strains. The resulting RIs from an analysis of variance that included components for recipient, donor, day of study, media, recipient × media, and donor × media (see Appendix) ranged from 0.4 to 0.59 (Table 3). Our main objective was to obtain a linear ordering (or map) of these mutations based on the RIs in Table 3, and taking into account the extent of their random variation and donor-versus-recipient symmetry of RIs. Analysis along these lines suggested a linear order of trf-2, trf-3, trf-1, trf-4, and trf-5, with trf-2 being further away from the others. Detailed statistical modeling and analysis are considered in the Appendix.

**LF⁻ mutations are linked.** Although only two LF⁻ mutants were isolated by streptomycin enrichment, naturally occurring LF⁻ mutants have been isolated (15) and several were included in this study. Transformation of mutant FA6353 (lrfl-1) with DNA from each of the other LF⁻ mutants yielded RIs varying from 0.07 to 0.57 (Table 4 and data not shown). Also shown in Table 4 are RIs calculated from an analysis of variance that included components for recipient, donor, and day. Similarly low RIs were obtained with LF⁻ strains FA1012, FA3000, and FA6411 as recipients (data not shown). Statistical analysis suggested that all but one of these mutants were linked (see Appendix). The unlinked LF⁻ mutation (lrfl-2) was a naturally occurring mutation, found in a derivative of gonococcal strain F62. Thus, we found two genetically distinct classes of LF⁻ mutations, all but one of which were clustered in a single linkage group.

**[TF LF⁻] mutations are linked.** Transformation of a [TF LF⁻]⁻ recipient strain (FA6303) with DNA from FA19 (TF⁺ LF⁺) yielded [TF LF⁻]⁺ transformants, but never TF⁺ LF⁻ or TF⁻ LF⁺ transformants. Furthermore, reverse studies with mutant FA6303 showed that the [TF LF⁻]⁺ phenotype reverted simultaneously to [TF LF⁻]⁻ at a frequency of about 10⁻⁶ (data not shown). Transformation of competent mutant FA6303 with DNA from 28 other [TF LF⁻]⁺ mutants produced RIs that varied from 0.1 to 0.5, indicating apparent linkage of the [TF LF⁻]⁺ mutations (data not shown). The linkage was confirmed by using isogenic strains FA6303, FA6370, FA6378, FA6381, and FA6382 in reciprocal trans-

### Table 3. RIs of reciprocal transformations between TF⁻ mutants based on ANOVA estimation

| Recipient strain | FA6356 trf-2| FA6342 trf-3| FA6338 trf-1| FA6367 trf-4| FA6366 trf-5 |
|------------------|-------------|-------------|-------------|-------------|-------------|
| FA6356 trf-2     | 0.07 (4)    | 0.08 (4)    | 0.14 (4)    | 0.24 (4)    |             |
|                  | [0.04, 0.13] | [0.04, 0.14]| [0.08, 0.26]| [0.13, 0.44]|             |
| FA6342 trf-3     | 0.04 (4)    | 0.14 (4)    | 0.14 (4)    | 0.33 (4)    |             |
|                  | [0.02, 0.07]| [0.07, 0.25]| [0.08, 0.26]| [0.18, 0.62]|             |
| FA6338 trf-1     | 0.05 (7)    | 0.09 (6)    | 0.24 (5)    | 0.42 (6)    |             |
|                  | [0.03, 0.07]| [0.05, 0.14]| [0.14, 0.40]| [0.26, 0.69]|             |
| FA6367 trf-4     | 0.13 (4)    | 0.41 (4)    | 0.13 (3)    |             |             |
|                  | [0.07, 0.25]| [0.22, 0.78]| [0.30, 1.19]| [0.06, 0.25]|             |
| FA6366 trf-5     | 0.09 (4)    | 0.26 (5)    | 0.21 (5)    |             |             |
|                  | [0.05, 0.16]| [0.15, 0.44]| [0.21, 0.59]| [0.12, 0.35]|             |

* Donor strain.
* 95% confidence intervals are given in brackets.
formations. The RIs were identical regardless of whether TF or LF was used to select transformants.

**55**Fe uptake by mutants. Fe-starved cells of FA6367 (TF−) demonstrated no net uptake of TF-bound 55Fe as compared with Fe-starved FA6367 was able to acquire normal levels of 55Fe from both LF and citrate (Fig. 1). Each of the other tested TF− mutants (FA6338, FA6342, FA6356, and FA6366) gave essentially identical results (data not shown). Fe-starved cells of FA6353 (LF−) demonstrated insignificant (<1%) uptake of LF-bound 55Fe as compared with FA19, but retained wild-type ability to internalize both TF- and citrate-bound 55Fe (Fig. 1). Fe-starved cells of [TF LF]− mutant FA6303 were reduced dramatically in the ability to internalize both TF- and LF-bound 55Fe, but retained normal ability to take up citrate-bound 55Fe (Fig. 1). Thus, each mutant class demonstrated nearly total loss of 55Fe uptake for either TF or LF, or both, in keeping with the results of growth assays; the mutations had no effect on Fe acquisition from other Fe sources.

**Identification of a gonococcal TF receptor.** In an equilibrium TF-binding assay, Fe-starved FA19 (TF+) demonstrated saturable specific binding of [125I]TF. The level of TF receptor activity was dramatically reduced during Fe-replete growth conditions (Fig. 2). Scatchard analysis of the data in Fig. 2 revealed a copy number of approximately 2,800 receptors per CFU, with an apparent affinity (Kd) of 0.46 μM (data not shown). Neither mouse TF nor human LF competed with the binding of human TF (data not shown). In contrast, apo-TF appeared to compete almost as effectively as Fe-TF: the difference in competitive binding between apo-TF and Fe-TF was not statistically significant after four experiments (Fig. 3). In a solid-phase (dot blot), TF-binding assay, human LF and mouse TF did not compete with the Fe-TF-HRP conjugate, whereas both apo-TF and Fe-TF did compete strongly (Fig. 4). These results confirmed the

![FIG. 1. Rate of 55Fe uptake by Fe-starved cells of wild-type strain FA19 (○), TF− mutant FA6367 (□), LF− mutant FA6353 (●), and [TF LF]− mutant FA6303 (■). Uptake values represent the difference in amount of 55Fe internalized by energy-sufficient cells and energy-poisoned (KCN-treated) cells. Each value represents the mean of two or more experiments ± standard deviation.](image1)

![FIG. 2. Binding of [125I]TF by Fe-starved cells of wild-type strain FA19 (○), Fe-sufficient cells of FA19 (△), and Fe-starved cells of TF− mutant FA6366 (○). Values represent the mean of three or more experiments ± 1 standard deviation.](image2)

| DNA donor strain | Observed (no. of exp) | Estimated* |
|------------------|------------------------|------------|
| FA1012 (lrf-3)   | 0.09 (5)               | 0.05       |
| FA1092 (lrf-5)   | 0.10 (3)               | 0.06       |
| FA1095 (lrf-6)   | 0.2 (3)                | 0.08       |
| FA3000 (lrf-4)   | 0.07 (4)               | 0.06       |
| FA3002 (lrf-7)   | 0.15 (5)               | 0.10       |
| FA6246 (lrf-2)   | 0.57 (5)               | 0.40       |

*Recipient strain, FA6353 (lrf-1).

*Values were determined from LF+ and Rif transformants.

*See Table 2, footnote d.
existence of a saturable, low-affinity, gonococcal TF receptor, as suggested previously (11). The receptor appeared to bind human TF specifically, but did not appear to be able to discriminate effectively between Fe-TF and apo-TF.

Because many eucaryotes possess TF receptors, we considered that these receptors could be similar in structure to the gonococcal TF receptor. Antibodies against the human or chicken TF receptors did not react in Western blots (immunoblots) with Fe-starved gonococcal membranes or in colony immunoblots with Fe-starved whole gonococcal cells; likewise, a cDNA clone of the human TF receptor (obtained from F. Rudde, University of California at San Francisco) failed to hybridize to gonococcal DNA under low stringency (data not shown).

LF binding was not characterized by the equilibrium-phase experiments because of excessive background due to the sticking of LF to the plastic microcentrifuge tubes. Analysis of the mutants and the parent strain by the solid-phase assay suggested that gonococci also possess an LF receptor (see below).

TF mutants are deficient in TF-receptor function. In a dot blot TF-binding assay (using ligand dilutions), TF mutants FA6338, FA6356, FA6342, FA6367, and FA6366 all were dramatically reduced in ability to bind TF-HRP as compared with the wild type, parental strain FA19 (Fig. 5). These mutants were unaltered in ability to bind LF-HRP (Fig. 6). In a quantitative, equilibrium-phase binding assay with live cells, FA6366 was reduced significantly in its ability to bind [125I]TF as compared with the wild type (Fig. 2), suggesting that the differences in binding of the HRP conjugates to dried cells reflected physiologically important reduction in binding of the ligand.

In quantitative, equilibrium-phase binding assays, Fe-starved TF mutants bound similar amounts of [125I]TF as compared with Fe-sufficient FA19 cells (Fig. 2). One explanation of this phenomenon would be that Fe-starved TF mutants are reduced in receptor number, perhaps due to a deficient regulatory mechanism for the TF receptor. Alternative explanations are that the Fe-starved TF mutants bind less ligand due to the lower affinity of an altered TF receptor or that there exists a second low-affinity or low-copy-number receptor in which the mutants are not defective. Scatchard analysis of data from [125I]TF-binding experiments, using Fe-starved FA19, did not reveal a second receptor (data not shown). Iron regulation of receptor function was tested in the following experiment. In a dot blot binding assay (using cell dilutions), Fe-starved mutant FA6342 (TF-) bound TF approximately as well as Fe-sufficient FA19 (Fig. 7), as above. However, Fe-starved cells of FA6342 were able to bind TF-HRP significantly better than FA6342 cells which were grown under Fe-sufficient conditions (Fig. 7). The relative upregulation in activity was similar to the upregulation of receptor activity in FA19. This experiment demonstrated that the mutant fully retains iron regulation.

LF mutants are deficient in LF-receptor function. In dot

**FIG. 3.** Binding of [125I]TF (5 μM) by live Fe-starved cells of FA19, in the presence of increasing concentrations of unlabeled Fe-TF (C) or Apo TF (C). Values represent the mean of two experiments performed in duplicate.

**FIG. 4.** Binding of TF-HRP (500 ng/ml) to Fe-starved cells of FA19, in the presence of decreasing concentrations of unlabeled human Fe-TF, human ApoTF, mouse Fe-TF, or human LF (nanograms per milliliter).

**FIG. 5.** Binding of TF-HRP to Fe-starved cells of wild-type strain FA19; TF mutants FA6338, FA6356, FA6342, FA6367, and FA6366; LF mutant FA6353; and [TF LF] mutant FA6303. Concentrations of TF-HRP (nanograms per milliliter) are shown above each column.
Receptor-mediated acquisition of Fe from human TF and LF has been suggested for several human specific pathogens, including the gonococcus (11, 23–25, 32, 33). We further characterize the gonococcal TF and LF receptors and provide substantial genetic and biochemical evidence for the critical importance of the TF and LF receptors to Fe acquisition from these sources.

The human Fe-binding glycoproteins TF and LF are very similar in structure and affinity for Fe. Not surprisingly, the mechanisms used by the gonococcus to scavenge Fe from these sources share several characteristics, including the use of receptors. However, the gonococcal TF and LF receptors are distinct entities. Mutants blocked in TF receptor function retained wild-type ability to bind LF and vice versa. Reciprocal transformations between TF− and LF− mutations always produced an RI of 1.0 or greater, indicating nonlinkage of the respective mutations and thus nonlinkage of genes responsible for TF and LF receptor function. Point mutations which simultaneously disrupted Fe acquisition from both TF and LF did not affect receptor function and were not linked to those mutations which did disrupt the binding of TF or LF.

The published affinity of the gonococcal TF receptor is quite low ($K_d$, $5 \times 10^{-7}$ M) compared with the eucaryotic TF receptor ($K_d$, $10^{-9}$ M) (34). However, the affinity of the gonococcal receptor is very similar to that reported for the meningococcal TF receptor (33). The gonococcal receptor also resembles the meningococcal receptor (27, 33) in its inability to discriminate effectively between Fe-TF and apo-TF. This is also quite unlike the TF receptor of humans which at physiological pH binds apo-TF at a much reduced affinity compared with Fe-TF. For these reasons, it may appear remarkable that the gonococcus is able to compete effectively for essential Fe-TF in an environment in which Fe-TF is bound more avidly and specifically by host cells. However, the gonococcal TF receptor may not directly compete with host TF receptors, since the concentration of unbound TF in serum approaches 30 μM, far above the 1 μM required to saturate binding to the gonococcus (14). The relatively low affinity of the receptor for TF and saturation at concentrations well below serum levels may also explain why it is not necessary for the gonococcus to discriminate between Apo-TF and Fe-TF: each TF molecule readily dissociates from the gonococcal cell surface and is rapidly replaced by another TF molecule.

The TF receptor appears to be essential to Fe scavenging from TF. Point mutants which lost the wild-type ability to bind TF were unable to internalize TF-bound Fe and consequently were unable to grow when TF was the sole Fe source. Similarly, point mutants which could not bind LF were not able to utilize it as an Fe source. These results suggest that receptor binding is a prerequisite to Fe scavenging from both TF and LF and further suggests that receptor-independent pathways for Fe acquisition from these sources do not exist. It is not clear whether the trf and lrf mutations affect the receptor genes, or whether these mutations disrupt the function of the receptors by altering the framework in which the receptors lie. The framework could include various outer membrane proteins, lipopolysaccharides, or other membrane components. Further work clearly is required to determine whether trf and lrf mutations lie within the structural genes for the respective receptors.

The TF and LF utilization pathways appear to be linked...
by the common use of at least one protein not involved in receptor function. The [TF LF]− mutants simultaneously lost the ability to utilize TF and LF, but retained the ability to bind both of these proteins. These mutants retained the ability to utilize all other Fe sources, suggesting that the protein common to the TF and LF utilization pathways was not required for Fe3+ uptake from citrate or Fe3+ uptake from heme compounds. The function altered by the iuf mutations is unknown, but the iuf-encoded protein may be required for releasing Fe from TF or LF bound to receptors or for transport of Fe into the cells after release from TF or LF. The specificity of iuf mutations for Fe uptake from TF or LF clearly distinguishes them from the mutation isolated in meningococcal strain FAM11, in that FAM11 was unable to take up any form of Fe3+ (6). The very close similarity of the Fe-scavenging systems of gonococci and meningococci suggests that this and presumably many other classes of gonococcal Fe utilization mutants remain to be discovered.

The recombination data from reciprocal crosses between gonococcal Fe uptake mutants suggested the existence of three linkage groups: one for trif mutants, a second for lfr mutants, and a third for iuf mutations. Within each group, some mutations appeared to be very close on the genome (RI, 0.05 to 0.1); whereas others appeared to be more distant (RI, 0.4 to 0.6). Unfortunately, there are few data by which to relate genetic distances to physical map distances in the gonococcus. In an earlier report, recombination data were related to physical sizes of transforming DNA for certain linked gonococcal ribosomal genes; loci that exhibited 10 to 20% cotransformation were estimated to be separated by 10 to 15 kilobases (30). This suggests that mutations that exhibit an RI of 0.5 may be separated by several kilobases and conceivably could affect separate proteins. If this were so, it would suggest that the TF or LF receptors or both could be a complex of two or more proteins. Much more evidence is required to understand the physical proximity of these mutations and the nature of the affected gene products.

The isolation and characterization of the TF−, LF−, and [TF LF]− mutants have revealed that the disruption of a small number of proteins can effectively prevent gonococci from obtaining Fe from both TF and LF. If the ability to utilize TF and LF is critical to pathogenesis, similar disruptions caused by the binding of vaccine-directed antibodies may prove effective in preventing gonococcal infections. However, the importance of TF or LF utilization to gonococcal pathogenesis has not been clearly established. Although LF− mutants have been isolated in vivo, it is not clear how these gonococcal strains obtain essential Fe on mucosal surfaces where LF is the dominant Fe source. Leakage of serum TF or heme compounds to the mucosa, especially in menstruating women, may provide sufficient levels of Fe, whereas on rectal mucosa hydroxamate siderophores may be present in sufficient quantities to provide essential Fe (38). In contrast, the ability to utilize TF appears to be critical, as no TF− mutant has ever been isolated in vivo. Proof of the in vivo relevance of the TF and LF Fe-scavenging system may be possible by using these mutants in human challenge experiments.

APPENDIX

The data collected in transformation experiments were the number of CFU (Y) of N. gonorrhoeae that grew under various experimental combinations: different donor strains, different growth media selecting for LF+, TF+, and Rif+, and different recipient strains (for the reciprocal transformation experiment). The criterion of interest was a ratio of ratios, adjusting the colony count of the donor-recipient transformants in a test medium (LF+ or TF+) for the reference media (Rif+) and for the standard donor FA19-recipient transformant colony count. This is called the recombination index (RI). Comparisons of the various strain RIs were then made.

Statistical inferences for the RIs were based on a multifactor analysis of variance (ANOVA) (21). For such ANOVA methods, it is generally assumed that the chance variables incorporated in the usual statistical model (called the errors) have a closely normal distribution (symmetric about 0). However, in the current case, Y was a positive integer valued random variable, and it had presumably skewed distribution for which the usual normal distribution might not have been adequate. In practice, this drawback is often eliminated by the use of a transformation Y → Y* = a monotone function of Y. For count data, one may take Y* = log10Y or even Y*, for some λ > 0; the choice of λ = 1/2 is very common. Towards this objective, we adopted the following transformation: Y* = log10(colony count) = log10Y. For this transformed variate Y*, the assumed ANOVA model actually related to a multifactor multiplicative model for the Y, with the Y’s being all non-negative. However, the Y* are not restricted to be non-negative, and this simplifies the statistical analyses. For this log-dependent variate model, the errors were likely to be more symmetrically distributed (than for the multiplicative model), and moreover, log10(count) used in the analysis resulted in a reduced variation among the counts and simplified RI comparisons (the difference of log values is equivalent to standard ratios). Explanatory variables were donor strain type, medium type, day effect, and the interaction between strain and medium. The reciprocal transformation experiment had a more complex data structure due to different recipient strains, so the model corresponding to it was expanded to account for this source of variation and the strain-medium interaction within it (see below). The RI comparisons of interest were tested by using ANOVA contrasts of one or more linear combinations of the strain-medium interaction effects, which represent the across-strain variation of RIs. For the reciprocal transformation experiment, an additional analysis objective was the fitting of another statistical model which described the pattern of variation among the strain-medium interaction estimates of log10(RI) through a “map.” Thus, the experiment had a two-stage analysis — an overall analysis with a model for all sources of variation and embodied in it a model for the strain-medium interaction terms, which ultimately was interpreted as a map of the mutation sites.

Mapping the mutational sites. The purpose of statistical analysis in this experiment was to determine whether the RIs of the mutant strains displayed in Table 2 were statistically different from the standard FA19 (RI, 1.0 by definition) and from each other. An ANOVA model was fit with log10(colony count) as the dependent variable and strain type, medium type, their interaction, and day effect as explanatory variables. The day effect was found to be nonsignificant, and day was therefore removed from the model. Thus, the ANOVA model for the evaluation of RI was as follows: log10(counts) = strain + media + strain*media. The F-test for the interaction was highly significant (P < 0.001), which implied that some of the RIs might be significantly different from 1.0 and individually contrasted with FA19 LF+ and TF+ through F-tests for corresponding linear combinations of interaction effects. The RI for FA1012 LF+ was found to be significantly different from that for FA19 LF+ (P = 0.001), and FA6342 TF+ was found significantly different from FA19 TF+ (P = 0.004). Additional linear combinations of interaction effects were constructed for F-tests contrasting the LF+ and TF+ RIs of each mutant strain to one another. A significant difference was found for FA1012 and FA6342 (P = 0.001 and 0.002, respectively). The mutant strains were then compared with each other considering both TF+ and LF+ RIs. All three mutant strains were found to be statistically different from each other (P < 0.01). It could be concluded that the RI of FA1012 LF+ and the RI of FA6342 for TF+ were each significantly smaller than 1.0, but there was essentially no difference between FA6378 and FA19 for either LF+ or TF+.

LF− mutations are linked. Comparisons among the RIs for LF− mutations displayed in Table 5 were analyzed in a similar manner as described above. The ANOVA model of log10(colony count) in-
included the following explanatory variables: day effects (which were significant with \( P < 0.01 \)), strain type, medium type, and strain-medium interaction. The interaction was significant (\( P < 0.001 \)), indicating that some of the RIs might be different from 1.0, and further analyses were appropriate. F-tests for contrasts among linear combinations of interaction effects found the RIs for all strains except FA6246 significantly different from that for FA19 (\( P < 0.001 \)). Additional F-tests suggested that FA6246 was different from FA19 (\( P = 0.106 \)) and was different from all other mutant strains in Table 5. It could be concluded that all mutant strains except FA6246 had RIs significantly smaller than 1.0.

**TF** mutation sites are mapped. The objective of this experiment was to compare the RIs in Table 3 and use them to form a relational map of the mutant strains. This analysis was more complex due to the reciprocal nature of the transformations. Each strain was both a donor and a recipient, so the following effects were included in the ANOVA model of \( \log_{10}(\text{colony count}) \) as explanatory variables: recipient, donor within recipient, day within recipient, medium type, the interaction of recipient and medium type, and the interaction of donor and medium type within a recipient. The ANOVA model is as follows: \( \log_{10}(\text{counts}) = \text{recipient} + \text{donor(recipient)} + \text{day(recipient)} + \text{medium} + \text{recipient*medium} + \text{donor*medium} \) (recipient). All effects were significant at \( P < 0.001 \). The significant donor-medium within recipient interaction suggested that some of the corresponding RIs might be different from one another, and further analyses were appropriate. F-tests for contrasts among linear combinations of interaction effects found significant RI differences among all donor RIs for each recipient separately and for all recipients simultaneously (\( P \geq 0.001 \)). This implies that at least some strains, when in the role of donor, are significantly different from other donors, both when examining one recipient and taking all recipients into account.

The objective of further analysis was to construct a model which described the variation among the \( \log_{10}(\text{RI}) \) in terms of a map of mutation sites. This model had three features which were applied in three corresponding steps. These features were symmetry of the RI in Table 3 relative to the main diagonal, additivity of recipient and donor components, and equality of recipient and donor components. The structure imposed by the model is displayed in Table A1. F-tests for corresponding linear combinations of donor*medium within recipient interaction effects were applied to test each of the three steps leading to this map model. Although the results of these tests indicated significant lack of fit (\( P < 0.001 \)), the model was nevertheless considered descriptively reasonable because it explained about 65% of the variation among all the \( \log_{10}(\text{RI}) \).

As displayed in Table A1, the proposed model had five parameters (A through E) for characterizing the variation among RIs. Estimates of these parameters were determined by multiple linear regression methods. "Dummy" variables which represented the map structure parameters were defined, and then the following ANOVA model was fit: \( \log_{10}(\text{colony count}) = \text{recipient} + \text{donor(recipient)} + \text{day(recipient)} + \text{medium} + \text{recipient*medium} + \text{donor*medium} \) (recipient). All effects were significant at \( P < 0.001 \). The significant donor-medium within recipient interaction suggested that some of the corresponding RIs might be different from one another, and further analyses were appropriate. F-tests for contrasts among linear combinations of interaction effects found significant RI differences among all donor RIs for each recipient separately and for all recipients simultaneously (\( P \geq 0.001 \)). This implies that at least some strains, when in the role of donor, are significantly different from other donors, both when examining one recipient and taking all recipients into account.

The objective of further analysis was to construct a model which described the variation among the \( \log_{10}(\text{RI}) \) in terms of a map of mutation sites. This model had three features which were applied in three corresponding steps. These features were symmetry of the RI in Table 3 relative to the main diagonal, additivity of recipient and donor components, and equality of recipient and donor components. The structure imposed by the model is displayed in Table A1. F-tests for corresponding linear combinations of donor*medium within recipient interaction effects were applied to test each of the three steps leading to this map model. Although the results of these tests indicated significant lack of fit (\( P < 0.001 \)), the model was nevertheless considered descriptively reasonable because it explained about 65% of the variation among all the \( \log_{10}(\text{RI}) \).

### TABLE A1. Mutation map model structure

| Recipient strain | FA6338 | FA6342 | FA6356 | FA6366 | FA6367 |
|------------------|--------|--------|--------|--------|--------|
| FA6338           | A      | A      | A+B    | A+B+C  | A+B+C+D|
| FA6342           | A      | A+B    | A+B+E  | A+B+C+E| A+B+C+D+E|
| FA6356           | A+B    | A+B+E  | A+B+C  | A+B+C+E| A+B+C+D+E|
| FA6366           | A+B+C  | A+B+C  | A+B+C+E| A+B+C+D+E| A+B+2C+D+E|
| FA6367           | A+B+C+D| A+B+C+D+E| A+B+2C+D+E|          |        |

* A, A reference value; B, log(FA6342 RI) − log(FA6342 RI); C, log(FA6366 RI) − log(FA6366 RI); D, log(FA6367 RI) − log(FA6366 RI); E, log(FA6342 RI) − log(FA6338 RI), where the RIs are of the named recipients for all donors, or equally the named donors for all recipients.

### TABLE A2. Estimates of average RIs and (in parentheses) 95% confidence intervals for average RIs based on the reciprocal transformation map model

| Mutant strain | FA6338 | FA6342 | FA6356 | FA6366 | FA6367 |
|---------------|--------|--------|--------|--------|--------|
| FA6338        | 0.16   | 0.07   | 0.35   | 0.24   |        |
| FA6342        | (0.11,0.22) | 0.05   | 0.25   | 0.17   |        |
| FA6356        | (0.05,0.10) | (0.03,0.07) | 0.11 | 0.08   |        |
| FA6366        | (0.25,0.49) | (0.18,0.35) | (0.08,0.16) | 0.39 |        |
| FA6367        | (0.17,0.34) | (0.12,0.25) | (0.05,0.11) | (0.27,0.55) |        |

### TABLE A3. Reciprocal transformation map estimates of row/column distance ratios

| DNA strain | FA6338 | FA6342 | FA6356 | FA6366 | FA6367 |
|------------|--------|--------|--------|--------|--------|
| FA6336      | 0.43   | 0.31   | 0.28  | 0.20  |
| FA6342      | 2.31   | 0.72   | 0.66  | 0.45  |
| FA6356      | 3.22   | 1.40   | 0.92  | 0.63  |
| FA6366      | 3.52   | 1.52   | 1.09  | 0.69  |
| FA6367      | 5.13   | 2.22   | 1.59  | 1.46  |

* e.g., FA6356 RI is 0.43 times FA6342 RI regardless of recipient. All distance ratios are significant at the \( P = 0.05 \) level except the FA6338/FA6367 ratio of 1.09 (reciprocal = 0.92).
using multiplicative ratios whose reference value of no recombination between numerator and denominator is 1.0. This is in contrast to the more common recombination fraction (number of recombinants divided by the total number of matings for a particular donor and recipient) which results in a map of additive fractions whose reference value of no recombination between donor and recipient is zero.

We have previously commented on the three features which were applied in three corresponding steps to obtain Table A3. Also, we have noticed a significant lack of fit of the proposed model. Being aware of this lack of fit, we might consider an alternative (multiplicative) model with $Y^* = (\text{count})^\lambda Y^*$, for some $\lambda > 0$. A best-fitting model could have been chosen by estimating $\lambda$ from the data. However, the complexity of the statistical analyses would have increased enormously, and formulation of conclusions would not have been straightforward (with such a data-oriented version of $\lambda$). Thus, we prefer not to pursue such complexity. Larger data sets would enable further evaluation of statistical conclusions.

Further details on this analysis are available by request to the authors (S.M.D., G.G.K., and P.K.S.).

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI26837 (P.F.S.), AI15036 (P.F.S.), AI23357 (D.W.D.), and AI07001 (J.A.) from the National Institute of Allergy and Infectious Diseases.

We thank I. Trowbridge for providing antibodies against the human and chicken TF receptors; F. Ruddle for supplying a cDNA clone of the human TF receptor; A. Rountree and G. Gallagher for technical assistance; S. West for help with certain early experiments; S. Thompson for helpful discussions; and L. Brooks for the preparation of the manuscript.

LITERATURE CITED

1. Archibald, F. S., and I. W. DeVoe. 1980. Iron acquisition by Neisseria meningitidis in vitro. Infect. Immun. 27:322–334.
2. Biswas, G. D., S. A. Lacks, and P. F. Sparling. 1989. Transformation-deficient mutants of pilated Neisseria gonorrhoeae. J. Bacteriol. 151:77–82.
3. Bullen, J. J., H. J. Rogers, and E. Griffith. 1974. Bacterial iron metabolism in infection and immunity, p. 518–551. In J. B. Neilsen (ed.), Microbial iron metabolism: a comprehensive treatise. Academic Press, Inc., New York.
4. Catlin, B. W. 1973. Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitidis, and Neisseria lactamica in chemically defined media, and the use of the growth requirements in gonococcal typing. J. Infect. Dis. 128:178–186.
5. Dyer, D. W., W. McKenna, J. P. Woods, and P. F. Sparling. 1987. Isolation by streptomycin enrichment and characterization of a transferrin-specific iron uptake mutant of Neisseria meningitidis. Microb. Pathol. 3:351–363.
6. Dyer, D. W., E. P. West, W. McKenna, S. A. Thompson, and P. F. Sparling. 1988. A pleiotropic iron uptake mutant of Neisseria meningitidis lacks a 7-kilodalton iron-regulated protein. Infect. Immun. 56:977–983.
7. Eaton, U. W., P. Brandt, J. R. Mahony, and J. T. Lee. 1982. Haptoglobin: a natural bacteriostat. Science 215:691–692.
8. Ephrati Elizar, E., P. R. Srivasan, and S. Zamenhof. 1961. Genetic analysis, by means of transformation of histidine linkage groups in Bacillus subtilis. Proc. Natl. Acad. Sci. USA 47:56–63.
9. Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. Neisseria gonorrhoeae. 1. Virulence genetically linked to clonal variation. J. Bacteriol. 85:1274–1279.
10. Lacks, S., and R. D. Hotchkiss. 1960. A study of the genetic material determining an enzyme activity in Pneumococcus. Biochim. Biophys. Acta 39:506–517.
11. Lee, B. C., and A. B. Schryvers. 1988. Specificity of the lactoferrin and transferrin receptors in Neisseria gonorrhoeae. Mol. Microbiol. 2:827–829.
12. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:210–218.
13. Masson, P. L., J. F. Heremans, and C. H. Dive. 1966. An iron-binding protein common to many external secretions. Clin. Chem. Acta 14:729–734.
14. McKenna, W. R., P. A. Mickelsen, P. F. Sparling, and D. W. Dyer. 1988. Iron uptake from lactoferrin and transferrin by Neisseria gonorrhoeae. Infect. Immun. 56:785–791.
15. Mickelsen, P. A., E. Blackman, and P. F. Sparling. 1982. Ability of Neisseria gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from lactoferrin. Infect. Immun. 35:915–920.
16. Mickelsen, P. A., and P. F. Sparling. 1981. Ability of Neisseria gonorrhoeae, Neisseria meningitidis, and commensal Neisseria species to obtain iron from transferrin and iron compounds. Infect. Immun. 33:555–564.
17. Muller-Eberhard, U. 1970. Hemopexin. N. Engl. J. Med. 283:1090–1094.
18. Neilsand, S. B. 1981. Iron adsorption and transport in microorganisms. Annu. Rev. Nutr. 1:27–46.
19. Neilsand, S. B. 1981. Microbial iron compounds. Annu. Rev. Biochem. 50:715–731.
20. Neilsand, S. B. 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. 36:285–309.
21. Neter, J., W. Wasserman, and M. H. Kutner. 1985. Applied linear statistical models. Irwin, Homewood, Ill.
22. Norrod, P., and R. P. Williams. 1978. Growth of Neisseria gonorrhoeae in media deficient in iron without addition of siderophores. Curr. Microbiol. 1:281–284.
23. Peterson, K. M., and J. F. Alderete. 1987. Iron uptake and increased intracellular enzyme activity following host lactoferrin binding by Trichomonas vaginalis receptors. J. Exp. Med. 160:398–410.
24. Redhead, K., T. Hill, and H. Chart. 1987. Interaction of lactoferrin and transferrins with the outer membrane of Bordetella pertussis. J. Gen. Microbiol. 133:891–898.
25. Schryvers, A. B. 1988. Characterization of the human transferrin and lactoferrin receptors in Haemophilus influenzae. Mol. Microbiol. 2:467–472.
26. Schryvers, A. B., and L. J. Morris. 1988. Identification and characterization of the human lactoferrin binding protein from Neisseria meningitidis. Infect. Immun. 56:1144–1149.
27. Schryvers, A. B., and L. J. Morris. 1988. Identification and characterization of the transferrin receptor from Neisseria meningitidis. Mol. Microbiol. 2:281–288.
28. Simonson, C., D. Brener, and I. W. DeVoe. 1982. Expression of a high-affinity mechanism for acquisition of transferrin iron by Neisseria meningitidis. Infect. Immun. 36:107–113.
29. Sparling, P. F. 1966. Genetic transformation of Neisseria gonorrhoeae to streptomycin resistance. J. Bacteriol. 92:1364–1371.
30. Sparling, P. F., G. D. Biswas, and T. E. Fox. 1977. Transformation of the gonococcus, p. 155–176. In R. R. Roberts (ed.), the gonococcus. John Wiley & Sons, Inc., New York.
31. Stookey, L. L. 1970. Ferrozine—a new spectrophotometric reagent for iron. Anal. Chem. 42:779–781.
32. Tryon, V., and J. B. Baseman. 1987. The acquisition of human lactoferrin by Mycoplasma pneumoniae. Microb. Pathol. 3:437–443.
33. Tsai, J., D. W. Dyer, and P. F. Sparling. 1988. Loss of transferrin receptor activity in Neisseria meningitidis correlates with inability to use transferrin as an iron source. Infect. Immun. 56:3132–31328.
34. Ward, J. H. 1987. The structure, function, and regulation of transferrin receptors. Invest. Radiol. 22:74–83.
35. Weinberg, E. D. 1978. Iron and infection. Microbiol. Rev. 42:45–66.
In G. K. Schoolnik, G. F. Brooks, S. Falkow, C. E. Frasch, J. S. Knapp, J. A. McCutchan, and S. A. Morse (ed.), The pathogenic neisseriae. American Society for Microbiology, Washington, D.C.

37. West, S. E. H., and P. F. Sparling. 1985. Response of Neisseria gonorrhoeae to iron limitation: alterations in expression of membrane proteins without apparent siderophore production. Infect. Immun. 47:388–394.

38. West, S. E. H., and P. F. Sparling. 1987. Aerobactin utilization by Neisseria gonorrhoeae and cloning of a genomic DNA fragment that complements Escherichia coli fhuB mutations. J. Bacteriol. 169:3414–3421.