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Expression of the Type 1 Pneumococcal Pilus Is Bistable and Negatively Regulated by the Structural Component RrgA▽†

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The pneumococcal type 1 pilus, which is present in 25 to 30% of clinical isolates, has been associated with increased adherence and inflammatory responses and is being evaluated as a potential vaccine candidate. Here we show that expression of the pilus is bistable as a result of the molecular interaction between the transcription activator RrlA and a structural component of the pilus called RrgA. Sampling various clinical pneumococcal isolates that harbor the type 1 pilus-encoding islet, we show that distinct populations of cells can be identified with either undetectable or prominent pilus expression. When these two populations are separated and regrown in liquid medium, they are phenotypically different: the nonexpressing population reverts to the previous bimodal distribution, whereas the expressing population retains the same high level of pilus expression. Controlled exogenous expression of the regulatory pilus gene *rlrA* in a strain from which the endogenous version has been deleted increases pilus expression steadily, suggesting that the bistable expression of the pilus observed in wild-type cells is dependent on the native *rlrA* promoter. Finally, we demonstrate that RrgA is a negative regulator of pilus expression and that this repression is likely mediated through direct interaction with RlrA. We conclude that type 1 pilus expression in pneumococcus exhibits a bistable phenotype, which is dependent upon the molecular interplay between the RlrA and RrgA proteins. We suggest that this flexibility in expression may assist adaptation to a range of immune conditions, such as evasion of antipilus antibodies, within potential hosts.

The Gram-positive bacterium *Streptococcus pneumoniae* (pneumococcus), in which the genetic role of DNA was discovered (1), is among the most analyzed organisms. However, aspects of its pathogenicity continue to be elusive. Pneumococcus is one of the major causes of bacterial pneumonia, meningitis, and septicemia, accounting for about 11% of mortality worldwide in children under 5 years of age (31). The bacterium is a frequent colonizer of the nasopharynx in children; most children are colonized by pneumococcus at some point during the first 2 years of life. It remains unclear, however, why some children develop invasive disease, whereas in the majority of cases, colonization remains asymptomatic, and a combination of bacterial virulence and host factors may be responsible.

Currently available pneumococcal vaccines target the main virulence factor, the capsular polysaccharide, and generate systemic immunity via anticapsular antibodies (6, 7). Immunogenicity in infants requires that these polysaccharides be conjugated to carrier proteins, an approach that is practicable for only a minority of the >90 known capsular serotypes. These "conjugate" vaccines, however, have limitations, including selective coverage of capsular types included, only partial protection against mucosal disease, the phenomenon of serotype replacement, and high cost (14, 22). For these reasons, alternative vaccine strategies have been sought, including the development of serotype-independent protein-based vaccines (3, 8, 32).

One of the most recent identified antigens proposed as a potential vaccine target is the pneumococcal pilus (13). At least two types of pneumococcal pili exist: type 1, shown to have several properties related to colonization and invasive disease (4) and encoded by the *rlrA* pathogenicity islet present in 25% of strains (5); and a second, type 2, which is rarer (2). Both pili act as adhesins (2, 30); the type 1 pilus was shown to enhance colonization in a mouse model (4) and facilitate the formation of microcolonies and biofilms (28). Type 1-piliated pneumococcal strains were also shown to induce significantly more tumor necrosis factor alpha (TNF-α) in a mouse model of intraperitoneal sepsis than their pilus-negative isogenic controls (4).

The genes encoding the pneumococcal type 1 pilus are found in approximately 25% of clinical isolates (5). Different structural models of the type 1 pilus have been proposed (12, 16, 17, 34), but in general, it is agreed that the RrgB protein forms the backbone, to which are attached two ancillary proteins, RrgA and RrgC, which are thought to be an adhesin and anchor to the cell wall, respectively. Prior to universal immunization with pneumococcal conjugate vaccine Prevnar in the United States, the type 1 pilus was found predominantly in serotypes that were included in the vaccine (5). These serotypes, and thus the prevalence of pilus-positive strains, sharply declined after the introduction of this vaccine, but the percentage of pilus-positive strains returned to prevaccine levels within the subsequent 3 to 5 years (33).

This persistence of the pilus in the pneumococcal population despite the initial impact of immunization suggests that this

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TABLE 1. Characteristics of the strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
T4	Spontaneous Sm^r derivative of TIGR4	www.tigr.org
T4 Δ(pilus)	Δ*rlrA-srtD*::*cat* Sm^r Cm^r	This study
T4 Δ*rrgA*	Δ*rrgA*::Janus Km^r	This study
T4 Δ*rrgA* GFP	Δ*rrgA*::Janus Δ*rrgC*::*gfp*(*mut3*) Km^r	This study
T4 Δ*rrgC*	Δ*rrgC*::Janus Km^r	This study
T4 GFP	Δ*rrgC*::*gfp*(*mut3*) Sm^r	This study
T4 Δ*rlrAJ*	Δ*rlrA*::Janus Km^r	This study
T4 Δ*rlrA*	Δ*rlrA*::Sm^r	This study
T4 Δ*rlrA*$_c$ GFP	Δ*rlrA*::*cat* Δ*rrgC*::*gfp*(*mut3*) Sm^r Cm^r	This study
T4 Δ*rlrA*$_c$	Δ*rlrA*::*cat* Sm^r Cm^r	This study
T4 *rlrA*-VSV-G	*rlrA*-VSV-G Sm^r	This study
T4 Δ*rrgA rlrA*-VSV-G	*rlrA*-VSV-G Δ*rrgA*::Janus Km^r	This study
T4 Δ*malQ*	Δ*malQ*::Janus Km^r	This study
T4 GFP *Pmal-rlrA* Δ*rlrA*	Δ*rrgC*::*gfp*(*mut3*) *Pmal*Δ*malQ*::*rlrA* Δ*rlrA*::*cat* Sm^r Cm^r	This study
T4 GFP *Pmal23-rlrA* Δ*rlrA*	Δ*rrgC*::*gfp*(*mut3*) *Pmal23*Δ*malQ*::*rlrA* Δ*rlrA*::*cat* Sm^r Cm^r	This study
Plasmids		
pAC1000	Derivative of pEV3	15
pQE30-rrgA	Wild-type *rrgA* gene with His$_6$ tag	This study
pQE-30-rrgB	Wild-type *rrgB* gene with His$_6$ tag	This study
pET21b-rlrA-VSV-G	*rlrA* gene with VSV-G tag	This study

structure provides important selective advantages in colonization; at the same time, the fact that in all populations surveyed, the prevalence does not exceed 30% suggests that there must be a cost to the presence of the pilus, perhaps due to the age-dependent rise in antibodies directed against pilus type 1 proteins (33). This potential fitness cost led us to investigate the mechanism of regulation of pilus gene expression in pneumococcus. Here we show, using various clinical strains grown in different media, that the expression of the type 1 pilus is bistable, with cells within a population either expressing the pilus or not. Moreover, we show that the structural protein RrgA is a negative regulator of this expression via its interaction with the pilus regulator protein RlrA.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and plasmids are listed in Table 1. Primers used in this study are listed in the table in the supplemental material. Pneumococcal strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), on plates containing tryptic soy agar with 5% sheep's blood (TSA), or in minimal medium (19). Antibiotics (300 μg/ml kanamycin, 600 μg/ml streptomycin, and 4 μg/ml chloramphenicol) were added where indicated. *Escherichia coli* strains were grown in LB medium supplemented with 100 μg/ml ampicillin when indicated. All pneumococcal mutants were constructed from a spontaneous streptomycin-resistant (Sm^r) derivative of the serotype 4 clinical isolate TIGR4 (strain T4). Bacteria were killed by heating at 58°C for 1 h prior to flow cytometry analysis.

Cloning strategies. Pneumococcal mutants were generated using either a suicide vector, pAC1000, or by use of overlapping PCR strategies, as described previously (15, 18). Where indicated, mutants were made either using the bicistronic Janus cassette (36) or the chloramphenicol resistance gene. Every mutant reported in this study had the corresponding area of interest sequenced and its genotype confirmed.

A strain with full deletion of the pilus islet, T4 Δ(pilus), was constructed by PCR amplification of the upstream and downstream region of the *rlrA* pathogenicity islet with the Dop1/Dop2′ and Dop3′/Dop4 primer pairs, respectively. The upstream and downsream regions were combined by overlapping PCR and cloned into vector pGEM-T. The chloramphenicol resistance gene was amplified from plasmid pAC1000 using oligonucleotides (CmOp1/CmOp2) and further cloned in between upstream and downstream regions using the XbaI restriction site, which lead to the creation of the plasmid called pAL1. Finally, the product of the PCR using the Dop1/Dop4 primer pair on the pAL1 plasmid was used to transform the TIGR4 strain. Colonies that underwent the double-recombination event were selected on TSA plates supplemented with chloramphenicol, and their genotype was confirmed by PCR. Western blot analysis was then performed on cell wall extracts of T4 Δ(pilus) as described previously (20).

Strain T4 Δ*rrgA* was generated in the same fashion as strain T4 Δ(pilus) using primer pairs DrrgA1/DrrgA2, DrrgA3/DrrgA43, and DrrgA5/DrrgA6 (36). In order to evaluate the phenotype of a complemented Δ*rrgA* strain, the *rrgA* gene was reintroduced in its original locus.

In order to make some of the mutants needed for the study, genes *rrgC*, *rlrA*, and *malQ* were replaced by the Janus cassette independently and *rlrA* was replaced with the *cat* gene by an overlapping PCR strategy using the primers sets described in the table in the supplemental material. Strain T4 GFP was made by replacing the Janus cassette inserted instead of the *rrgC* gene with *gfp*(*mut3*), a *mut3* gene coding for green fluorescent protein (GFP). T4 GFP was then transformed with genomic DNA of T4 Δ*rlrA*$_c$ and T4 Δ*rrgA*; colonies were then selected on plates containing chloramphenicol or kanamycin, respectively, in order to isolate T4 Δ*rlrA*$_c$ GFP and T4 Δ*rrgA* GFP.

T4 Δ*rlrA* was made by removal of the Janus cassette previously inserted into the *rlrA* gene. Strain T4 *rlrA*-VSV-G was then made by inserting a vesicular stomatitis virus glyoprotein (VSV-G) epitope tag at the C terminus of *rlrA*. Strain T4 Δ*rrgA rlrA*-VSV-G was obtained by transforming strain T4 *rlrA*-VSV-G with genomic DNA of T4 Δ*rrgA* and selection using plates supplemented with kanamycin.

An *rlrA* mutant strain with *rlrA* under the influence of a maltose-inducible promoter was constructed as follows. T4 GFP *Pmal-rlrA* Δ*rlrA* was obtained in a succession of steps, by first transforming the T4 GFP strain with genomic DNA of T4 Δ*malQ* and selection on kanamycin. The resulting strain, T4 Δ*malQ* GFP, was further transformed with genomic DNA of T4 Δ*rlrA*$_c$ and a T4 Δ*rlrA* Δ*malQ* GFP strain was selected on a blood agar plate supplemented with chloramphenicol. Finally T4 GFP *Pmal-rlrA* Δ*rlrA* was obtained by replacing the Janus cassette with the *rlrA* gene. Because this strain showed expression of the pilus in THY, even in the absence of added maltose, suggesting leakiness of the promoter, the ribosome binding site (RBS) of the maltose promoter was altered by use of overlapping PCR in order to decrease the activity of the promoter. Several strains containing mutations of the RBS of the *malQ* promoter were evaluated by flow cytometry in the presence of increasing concentrations of maltose in minimal medium. Promoter *Pmal23* was selected as it had negligible pilus expression in the absence of maltose and increasing pilus expression with increasing concentration of maltose. Sequencing of the RBS of *Pmal23* showed that it contained TACGGA instead of ACGAGG present in the RBS of the wild-type T4 strain.

Protein purification and expression. Genes encoding His$_6$-tagged RrgA and RrgB were cloned into pQE30, whereas the gene encoding His$_6$-tagged RlrA-VSV-G was cloned into pET21b vector for expression. Plasmids were transformed into *E. coli* strain BL21(DE3) for expression. Bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.6 and then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 16°C. Bacteria were harvested and then suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1× antiprotease [Roche], 0.1% lysozyme, 10 mM $MgCl_2$, silicone, and DNase) before being sonicated six times for 30 s each. Samples were centrifuged for 30 min at 14,000 rpm in the cold, and supernatants were collected. Five hundred microliters of Ni-nitrilotriacetic acid (NTA) was added to samples, and the mixture was incubated for 1 h at 4°C. Samples were loaded onto a column, washed twice with buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole) at pH 8.0, and then eluted in buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole) at pH 8.0. Protein purity was analyzed on NuPage 4 to 12% Bis-Tris gel (Invitrogen), and protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay (Pierce).

Generation and testing of polyclonal antibodies. Purified recombinant His_6-tagged RrgA and RrgB were used to immunize guinea pigs and rabbits, respectively (Cocalico Biologicals, Inc.). Final antiserum bleeds were tested at various dilutions on pure protein to determine the optimal dilution for flow cytometry or Western blotting.

Flow cytometry analysis. Analysis was performed on a Beckman Coulter MoFlo Legacy flow cytometer. GFP and Alexa 488 were excited by a Coherent Sapphire solid-state laser emitting at 488 nm, whereas Alexa 660 was excited by a Dako red diode laser emitting at 635 nm. Prior to each experiment, the flow cytometer was aligned with Beckman Coulter flow-check Fluorospheres (part no. 6605359) and SPHEROUltra Rainbow calibration particles (catalog no. URCP-38-2K) and then configured to trigger off the side scatter (SSC) parameter. To confirm that the flow cytometer was capturing all of the bacteria via the SSC trigger, three sizes of Sphero polystyrene particles were run through the machine prior to each acquisition: 1.00 to 1.49 μm (catalog no. PP-10-10), 0.4 to 0.6 μm (catalog no. PP-01-10), and 0.2 to 0.3 μm (catalog no. PP-025-10). The flow cytometer settings remained the same for all experiments. Postacquisitional analysis was performed using Beckman Coulter Summit 4.3 software and Treestar FlowJo 8.7.3. Heat-killed bacteria were blocked for 1 h in buffer 0 (1× phosphate-buffered saline [PBS] with 1% bovine serum albumin [BSA]), treated for 1 h with primary antibody in buffer 1 (1× PBS, 0.05% Tween 20, 1% BSA), washed once with buffer 1, and then treated for 1 h with secondary antibody in buffer 1. Bacteria were washed three times in buffer 1 and analyzed by flow cytometry using MoFlo. The experiments were performed at room temperature. Alexa Fluor 488 or 660 secondary antibodies from Invitrogen were used at a 1/50 dilution.

Micrographs. Cells of *S. pneumoniae* T4 GFP, T4 Δ*rlrA* GFP, and T4 Δ*rrgA* GFP were suspended from TSA plates into 1 ml of PBS and fixed and imaged essentially as described previously (37).

Separation of bacteria by magnetic beads. To separate bacteria on the basis of pilus expression with magnetic beads, pneumococcal strains were grown to an optical density of 0.5. The equivalent of 5×10^7 CFU was blocked in buffer 0, treated for 1 h with an anti-RrgB antibody at a 1/200,000 dilution in buffer 1, and then washed twice in buffer 2 (buffer 1 with 2 mM EDTA). Bacterial pellets were suspended and incubated for 15 min at 4°C into 160 μl of buffer 2 and 40 μl of anti-rabbit IgG microbeads (Miltenyi Biotec). Bacteria were washed three times in buffer 2 and passed through MACS separation columns (Miltenyi). The flowthrough fraction was collected, and the absence of pilus expression was confirmed by flow cytometry or Western blotting. Eluted bacteria were harvested by centrifugation and examined by flow cytometry and Western blotting.

RNA isolation and real-time RT-PCR. Cells from 2 ml of bacteria at an OD_{600} of 0.5 were harvested and suspended in 100 μl of Tris-EDTA (TE) (Ambion). Cells were lysed on the amalgamator three times for 16 s after addition of glass beads (Sigma). Immediately afterwards, 350 μl of RLT buffer with β-mercaptoethanol (10 μl/ml) was added to samples, mixed, and then centrifuged for 2 min. Supernatants were collected, and 250 μl of ethanol (100%) was added. Subsequent steps were performed per the instructions included in the RNA minikit from Qiagen. Transcripts were quantified by quantitative real-time reverse transcription (RT)-PCR relative to the *gyrB* transcript essentially as described previously (23).

Coimmunoprecipation assay using purified proteins or live bacteria. Various amounts of RlrA-VSV-G and RrgA purified proteins were incubated together overnight at 4°C. The following day, 80 μl of buffer 3 (50 mM Tris-HCl [pH 7.8], 300 mM NaCl, 1 mM EDTA, 1% BSA, 2% Triton X-100) and 20 μl of agarose-immobilized goat anti-VSV-G antibody (Bethyl Laboratories) were added to each sample and incubated at room temperature for 1 h. After extensive washing in buffer 3, samples were suspended in SDS gel loading buffer, and Western blotting of these samples was performed for detection of RlrA-VSV-G and RrgA proteins. Protein bands were detected using either a rabbit anti-VSV-G affinity-purified antibody (Bethyl Laboratories, Inc.) or antibody against the RrgA protein.

To detect the association of the two proteins in the context of live bacteria, pneumococcal strains were grown in 30 ml of THY at an OD_{600} of 0.8. Bacterial cells were collected and lysed in buffer 4 (50 mM Tris-HCl [pH 7.8], 300 mM NaCl, 1× antiprotease [Roche], 1 mM EDTA, 2% Triton X-100) with 1% sodium deoxycholate and DNase. Samples were incubated for 30 min at 37°C and centrifuged, and supernatants were collected. Forty microliters of agarose-immobilized goat anti-VSV-G antibody was added to the samples, and the mixture was incubated overnight at 4°C. Samples were extensively washed in buffer 4 and suspended in SDS gel loading buffer. A Western blot analysis was performed to check for the presence of both RlrA-VSV-G and RrgA proteins.

ChIP. Cultures of T4 *rlrA*-VSV-G and T4 Δ*rrgA rlrA*-VSV-G were inoculated in triplicate from TSA plates at a starting OD_{600} of 0.05 and grown to an OD_{600} of 0.8 in THY broth. Chromatin immunoprecipitation (ChIP) was then performed using 3 ml of culture, and fold enrichment values were measured by quantitative PCR relative to the *gyrB* coding region essentially as described previously (9).

RESULTS

Expression of pneumococcal pilus type 1 is bimodal. In the course of studies focused on the analysis of the inflammatory response to pneumococci, we generated antibodies to the structural proteins of the pilus RrgA and RrgB and evaluated how these antibodies bound to the pneumococcal strain serotype 4 (T4) strain TIGR4 by flow cytometry. We began our studies by growing a clonal population of T4 in liquid medium, incubating it with RrgB polyclonal antibodies, and analyzing the results by flow cytometry. Surprisingly, and in contrast to findings by Moschioni et al. (27), a dual population was observed, with only roughly 30% of the population showing fluorescence following incubation with RrgB antibodies (Fig. 1A, left panel). We repeated this experiment now using the same protocol as described previously (27) and still detected two populations. The same observation was made when cells of strain T4 were grown in various media, such as tryptic soy medium, Dulbecco's modified Eagle's medium (DMEM), pneumococcal minimal medium, or RPMI (data not shown). For convenience, the low-fluorescence subpopulation will be referred as LPE (for low pilus expression) as opposed to HPE (high pilus expression) for the high-fluorescence subpopulation. When a strain deleted for the pilus genes was used, only cells of the LPE population remained, showing that the binding of the antibody was specific for the pilus protein in the HPE population (Fig. 1A, right panel). These findings were reproduced when antibodies directed against the other main pilus structural protein, RrgA, were used; the levels of expression of RrgB and RrgA correlated strongly with one another, as assessed by flow cytometry (Fig. 1B). We were not able to visualize the RrgC protein by flow cytometry, which may be due to its low abundance and/or location at the base of the pilus, potentially rendering it inaccessible to antibodies (29). Since deletion of the *rrgC* gene does not affect RrgB polymerization (11), we inserted the *mut3* gene encoding GFP [*gfp*(*mut3*)] at the site of the *rrgC* locus. We observed two populations of cells—one without and one with GFP expression (Fig. 1C, left panel). Overall, GFP expression correlates well with expression of the RrgB protein (Fig. 1C, right panel). This bimodality is not dependent on growth phase, as it was observed in cells harvested from blood agar plates or grown in liquid medium to the early, middle, or late log phase (data not shown). These data thus confirm that the type 1 pilus is expressed in a bimodal manner. Next we evaluated the pattern of pilus expression in a group of 13 clinical isolates

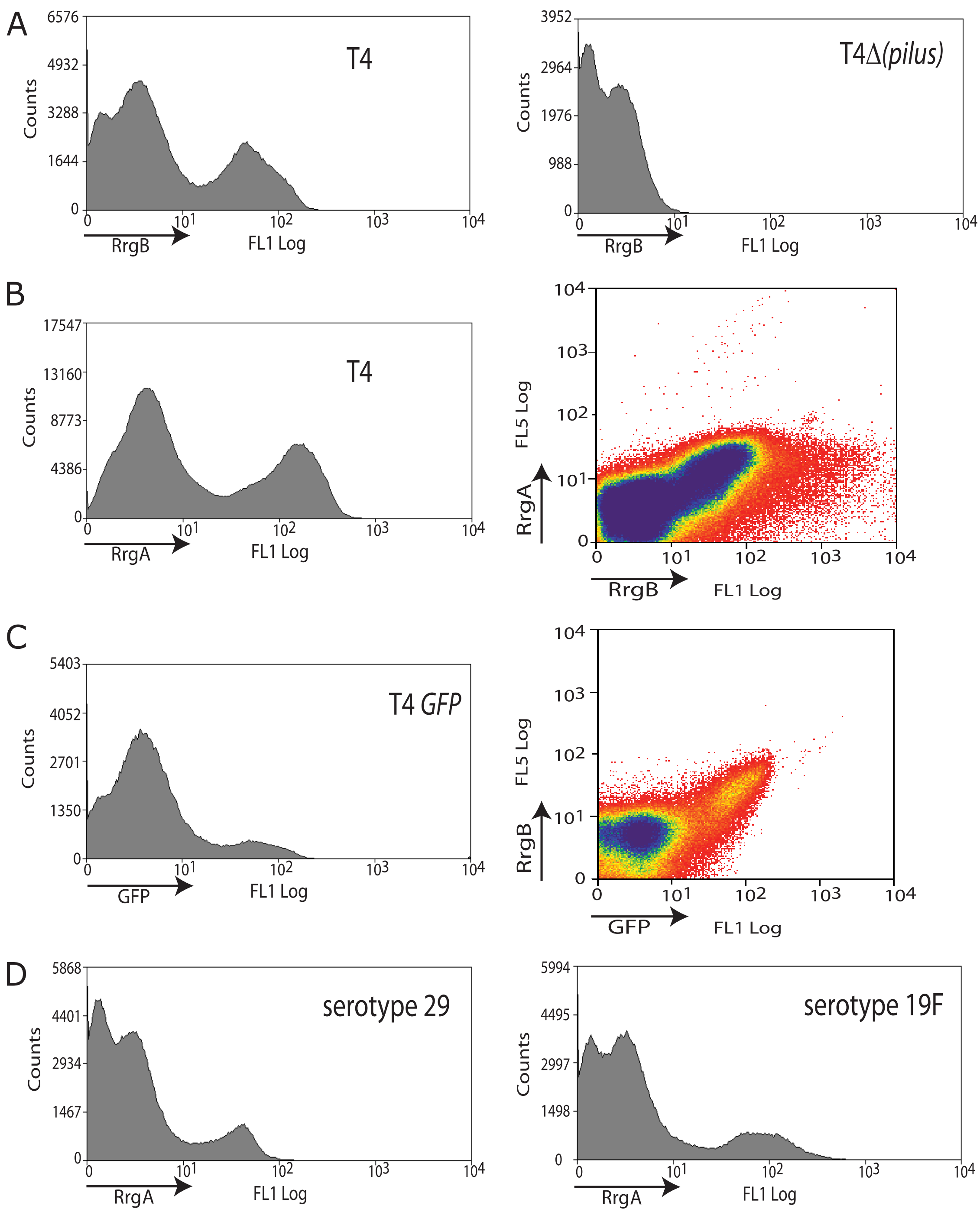

FIG. 1. The type 1 pneumococcal pilus is bimodally expressed. (A) Flow cytometry analysis performed on T4 and T4 Δ(pilus) using anti-RrgB antibody shows a dual population in the T4 strain as opposed to the T4 Δ(pilus) strain. The *x* axis represents the level of fluorescence, and the *y* axis represents the number of events counted. (B) Flow cytometry analysis of a T4 strain using anti-RrgA antibody also shows a bimodal population. When antibodies to both proteins were used to stain bacteria with different fluorophores (RrgA with Alexa 488 on FL1 and RrgB with Alexa 660 on FL5 [Invitrogen]), a strong and significant correlation was observed. (C) Flow cytometry of the T4 GFP strain shows that the GFP gene, replacing the *rrgC* gene, is also expressed bimodally. GFP expression and RrgB expression also correlate strongly in a T4 GFP strain when analyzed. (D) The pattern of pilus expression in various pneumococcal strains was examined, as detailed in the text; in the majority of cases (see text), bimodal distributions were apparent.

representing serotypes 4, 6B, 7C, 9V, 12F, 13, 14, 15F, 19A, 19F, 29, and 33F (strains derived from the collection described in reference 5) using an antibody to the RrgA protein (which is less variable than RrgB) (13). We observed the same type of dual population in 10/13 serotypes, most prominently in strains of serotypes 6B, 19F, and 29 (Fig. 1D). These results show that expression of the pilus is bimodal in strains other than T4.

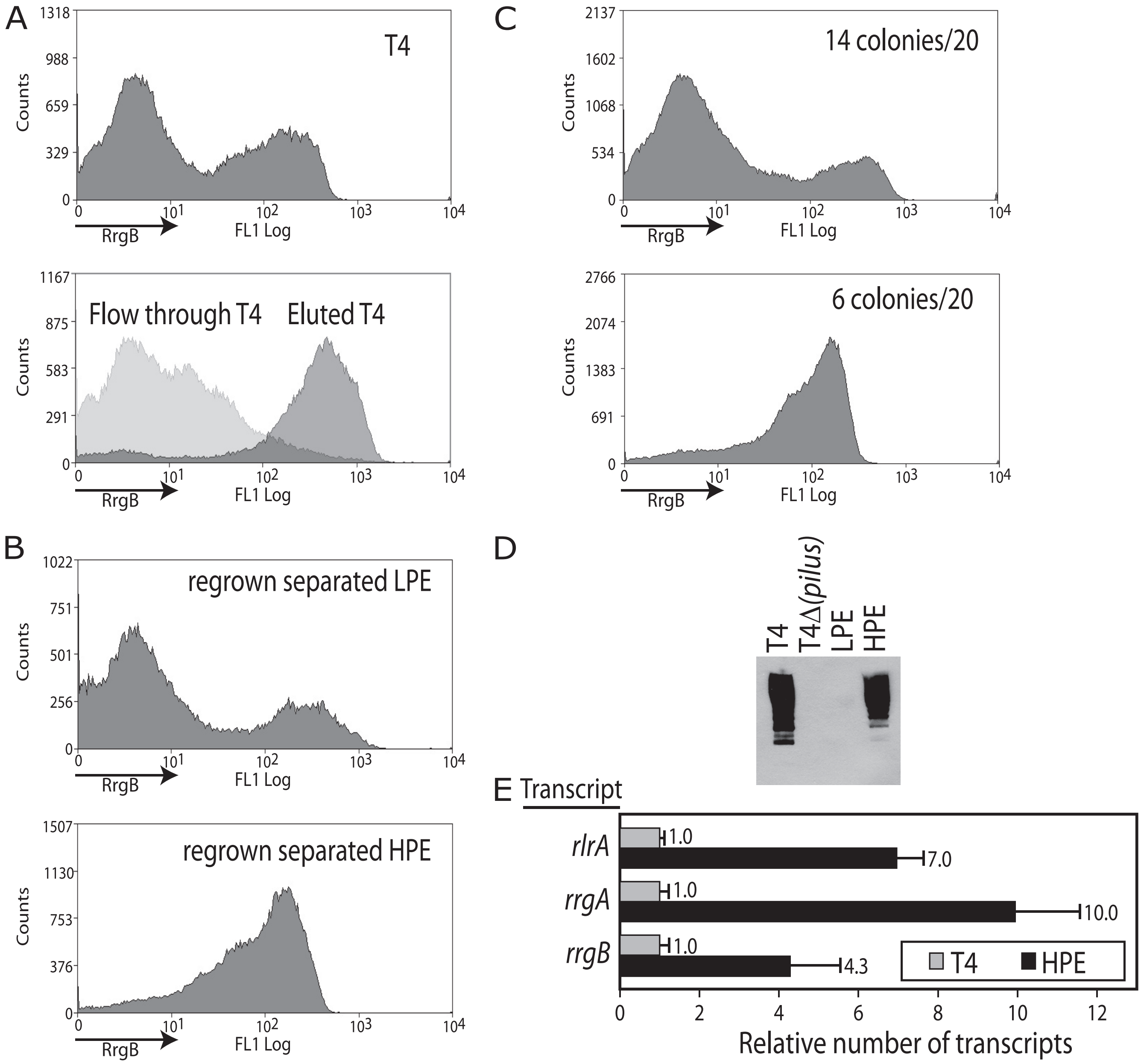

FIG. 2. The type 1 pilus is expressed in a bistable manner. (A) Regrowth of low- and high-pilus-expression (LPE and HPE, respectively) populations. (A) From a population of T4 cells exhibiting both LPE and HPE phenotypes, LPE and HPE cells were separated from one another using antibodies coupled with magnetic beads and immediately analyzed by flow cytometry targeting the RrgB protein. (B) Twenty colonies originating from either LPE or HPE cells were streaked on TSA plates and regrown in THY. The data presented here are representative of the 20 results obtained. LPE reverts to the original bimodal population, whereas the HPE phenotype remains constant. (C) Pattern of pilus expression from 20 isolated colonies from a T4 strain population. (D) Western blot analysis of flowthrough (LPE) and eluted (HPE) cells using anti-RrgB antibody confirms the absence of RrgB protein in LPE populations, in contrast to its presence in HPE cell populations. Note that the concentrations of bacterial cell lysates used in T4, HPE, and LPE lanes were not adjusted, so that relatively more protein from LPE cells was loaded on the gel; despite this, no RrgB band can be observed in the LPE lane. (E) Real-time RT-PCR of T4 and HPE populations performed to evaluate the quantity of *rlrA*, *rrgA*, and *rrgB* transcripts relative to *gyrB* transcript. The abundance of transcripts for the three genes analyzed in cells of a T4 strain was arbitrarily set to 1. The results shown here are representative of the three experiments performed. The expression of the *rlrA*, *rrgA*, and *rrgB* genes is significantly increased in HPE cells compared to cells of a T4 strain.

Type 1 pilus gene expression is bistable. To further evaluate these findings, we separated the LPE and the HPE cell populations using magnetic beads. A clonal population of T4 cells was grown to mid-exponential phase and incubated with antibodies to RrgB, following which, the bacteria were incubated with anti-rabbit IgG-coupled magnetic beads; after incubation, the antibody-bound and non-antibody-bound bacteria were separated by elution in the presence or the absence of a magnetic field, as described in Materials and Methods. Figure 2A shows the distribution of the original bacterial suspension (top panel) and the flowthrough (light gray, bottom panel) (LPE) and eluted bacteria (dark gray, bottom panel) (HPE) following this procedure. Interestingly, when cells of the LPE population were plated and 20 of the resultant colonies were subsequently grown in liquid medium once to mid-log phase, the bimodal pattern of pilus expression was restored (reflecting about 70% LPE and 30% HPE cells) (Fig. 2B), demonstrating that the switch to the HPE phenotype can occur very rapidly. In contrast, similar growth of the HPE population did not result in a detectable decrease in pilus expression, with the majority of cells retaining the HPE phenotype. This was also the case when these HPE cells were regrown and plated multiple times; at most, only a small percentage of the population appeared to revert to the LPE state (data not shown). When cells from 20 individual colonies of our TIGR4 reference strain were each grown to mid-log phase in liquid medium, cells in 14 out of 20 (70%) of the cultures exhibited the same bimodal pattern as cells of the original strain, whereas cells in the remaining cultures (30%) were predominantly of the HPE phenotype (Fig. 2C). Thus, while cells in the LPE population gradually revert to a bimodal distribution upon regrowth, the phenotype of the HPE population does not revert at the same rate. Western blotting performed on isolated LPE and HPE populations using anti-RrgB antibodies confirmed that the pilus is not detectable in LPE bacteria, in contrast to HPE bacteria (Fig. 2D), confirming the findings by flow cytometry. The differences in pilus expression can also be noted at the transcriptional level; real-time RT-PCR of regrown HPE bacteria demonstrates strong upregulation of *rlrA* and genes encoding the structural proteins RrgA and RrgB compared to the TIGR4 reference strain (Fig. 2E). These findings demonstrate that cells of the LPE population can upregulate pilus expression such that the total population reaches the previously observed equilibrium, whereas HPE cells tend to be fixed in this phenotypic state. Sequence analysis of the region encompassing *rlrA* and *rrgA* in HPE cells did not show any differences from the wild-type sequence, showing that the HPE phenotype is not the result of genetic mutation.

In summary, our data show that two distinct phenotypes of pilus expression (LPE and HPE) can be detected following growth of a clonal population of pneumococci and, furthermore, that when LPE (but not HPE) cells are isolated, their subsequent growth results in the restoration of a bimodal pilus expression phenotype. Thus, we conclude that expression of the type 1 pilus is bistable.

The bistable phenotype is dependent on the endogenous *rlrA* promoter. Next, we tried to identify potential mechanisms for pilus bistability. Since RlrA has been shown to be the major positive regulator of type 1 pilus expression (15), we evaluated whether bistability was mediated through the *rlrA* promoter. As expected, a strain in which the *rlrA* gene was deleted does not express the pilus (Fig. 3A). These results were confirmed using strain T4 Δ*rlrA* GFP—no fluorescence was observed (Fig. 3B3 and B4). To evaluate whether bistable expression is mediated by the native *rlrA* promoter, we constructed a strain in which the endogenous *rlrA* gene was deleted and an exogenous *rlrA* gene was added under the control of a modified maltose promoter. As shown in Fig. 3C, the increasing concentrations of maltose in minimal media were associated with a gradual and steady increase in pilus expression but without any evidence of a bimodal distribution. No effect of maltose could be detected on the T4 strain (data not shown). These data support the hypothesis that bistable expression of the pilus is dependent upon having the endogenous *rlrA* promoter driving *rlrA* expression.

RrgA is a negative regulator of pilus expression. In the course of evaluating the role of RrgA in inflammatory responses, we had noted that a deletion of the *rrgA* gene from T4 resulted in a significant increase in the expression of the pilus as determined by Western blotting; furthermore, the level of RrgB expression in the *rrgA* mutant resembled that found in HPE cells (Fig. 4A). Thus, we hypothesized that RrgA may be a negative regulator of pilus expression. When we analyzed the patterns of pilus expression of 20 independent T4 Δ*rrgA* mutants by flow cytometry, we found that every isolate displayed the HPE phenotype (Fig. 4B), as was also noted by microscopy when a strain deleted for the *rrgA* gene and carrying the GFP gene (T4 Δ*rrgA* GFP) was examined (Fig. 3B5 and B6). Complementation of *rrgA* restored the bimodal pattern of expression (Fig. 4C), thus supporting the hypothesis that RrgA negatively regulates bistable expression of the type 1 pilus.

Flow cytometry and Western blotting of T4 *rlrA*-VSV-G and T4 Δ*rrgA rlrA*-VSV-G mutants confirmed that the pilus expression was not modified by the presence of the tag. We then performed a chromatin immunoprecipitation (ChIP) experiment comparing cells of wild-type T4 *rlrA*-VSV-G and T4 Δ*rrgA rlrA*-VSV-G mutant strains and showed that the deletion of the *rrgA* gene results in a significant increase in amount of RlrA associated with the *rlrA-rrgA* intergenic region, whereas there was no evidence of increased association of the RlrA protein with either the *rrgB* or *srtB* promoter regions (Fig. 4D). These results raised the possibility that RrgA may mediate its negative effects on pilus gene expression by interacting directly with RlrA, a positive regulator of pilus gene expression. We therefore tested whether RlrA and RrgA could interact with one another by immunoprecipitation. We mixed the two purified proteins RlrA-VSV-G and RrgA and incubated the mixture with agarose-immobilized goat anti-VSV-G antibody. As shown in Fig. 4E (left panel), Western blotting with an anti-RrgA antibody confirmed that RrgA coprecipitates with RlrA, whereas no RrgA could be detected in the absence of RlrA. A similar finding was observed with lysate obtained from pneumococci expressing wild-type RrgA and a VSV-G-tagged version of RlrA, strongly supporting the idea that the two proteins interact (Fig. 4E, right panel). Thus, our data suggest that the bistable expression of the pilus is a consequence of the molecular interaction between RlrA and the negative regulator RrgA. We suggest that this negative regulation results in less RlrA at the *rlrA* promoter.

DISCUSSION

Gram-positive pili differ from their Gram-negative counterparts by the presence of covalently linked subunits containing a conserved LPXTG motif (or a variant thereof), which is the target of sortase enzymes that catalyze the covalent attachment of the backbone pilins to the cell wall. Published data suggest that the role of pili in Gram-positive bacteria in colonization and disease may be both species and process dependent. In group B streptococcus (GBS) and group A streptococcus

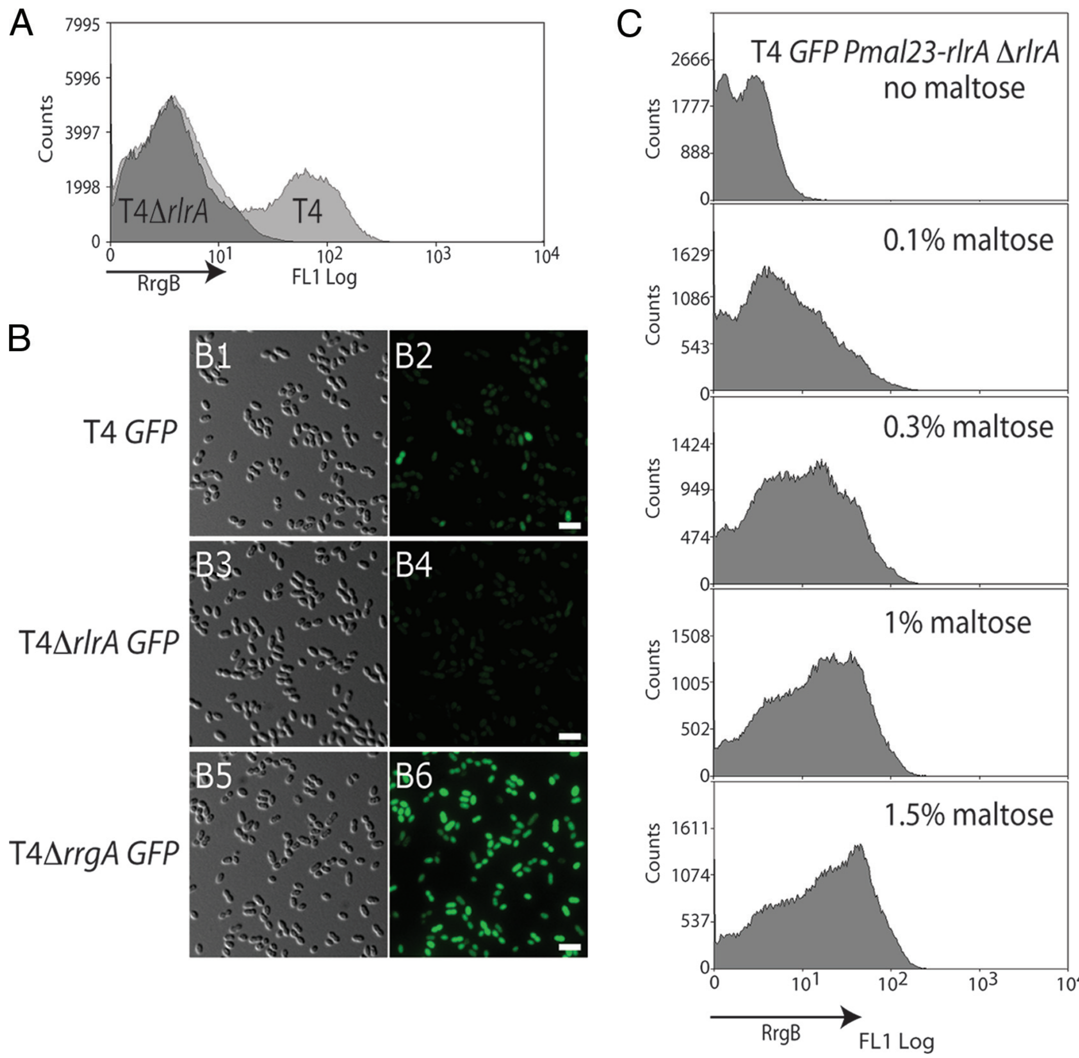

FIG. 3. The native *rlrA* promoter is essential for bistable expression of the pilus. (A) Comparison of pilus expression levels in cells of the T4 Δ*rlrA* strain and wild-type T4 strain by flow cytometry using anti-RrgB antibodies. (B) Fluorescent microscopy of T4 GFP, T4 Δ*rlrA* GFP, and T4 Δ*rrgA* GFP strains. Fluorescence microscopy of the T4 GFP strain (B1 and B2), in which the *rrgC* gene was replaced by the *mut3* gene coding for GFP [*gfp*(*mut3*)], confirms bistability of pilus expression. In contrast, fluorescence microscopy of T4 Δ*rlrA* GFP confirms the absence of pilus expression in the mutant (B3 and B4), whereas GFP expression is noted in all T4 Δ*rrgA* GFP cells (B5 and B6). (C) Expression of the pilus in cells of strain T4 GFP *Pmal23-rlrA*, in which the endogenous *rlrA* gene was deleted and an exogenous *rlrA* gene was added under the control of an altered maltose promoter. A gradual but monophasic increase in pilus expression is observed.

(GAS), as well as pneumococcus, pili are shown to be adhesins that contribute to enhanced epithelial cell attachment. However, whereas in GBS and pneumococcus the pilus appears to contribute to virulence in systemic disease (4, 24, 25), the opposite was found in *Streptococcus pyogenes*, where, unexpectedly, the presence of the pilus promotes capture of the organism in extracellular traps, thereby reducing the virulence potential of the organism (10). From an epidemiological perspective, it is also unclear whether the pilus contributes to systemic virulence in pneumococcus, as there is no difference in prevalence of pilus genes in nasopharyngeal and invasive disease isolates (5).

The data presented here add another level of complexity to the issue. Pilus expression in pneumococci appears to be tightly regulated, with only a subset of bacteria expressing the pilus at any one time. This phenotypic variability is critically dependent on the RrgA protein, one of the structural proteins of the pilus, which physically interacts with the positive regulator RlrA, thus repressing pilus gene expression and contributing to the bistable phenotype that we observed.

Bistability is a mechanism by which bacteria can introduce heterogeneity within an isogenic population of cells, which may provide a selective advantage for subsets of bacteria under adverse conditions and which has been described in many different organisms (39). For example, some bacteria enter a dormant or vegetative state to resist antibiotic treatment, a process that would otherwise require the development or acquisition of antibiotic resistance genes (21). Bistable expression of several virulence factors may be beneficial to an organism as it may create subpopulations of cells within an isogenic population that differ in their adaptation to the environment (38). We believe the bimodal pattern of pilus expression de-

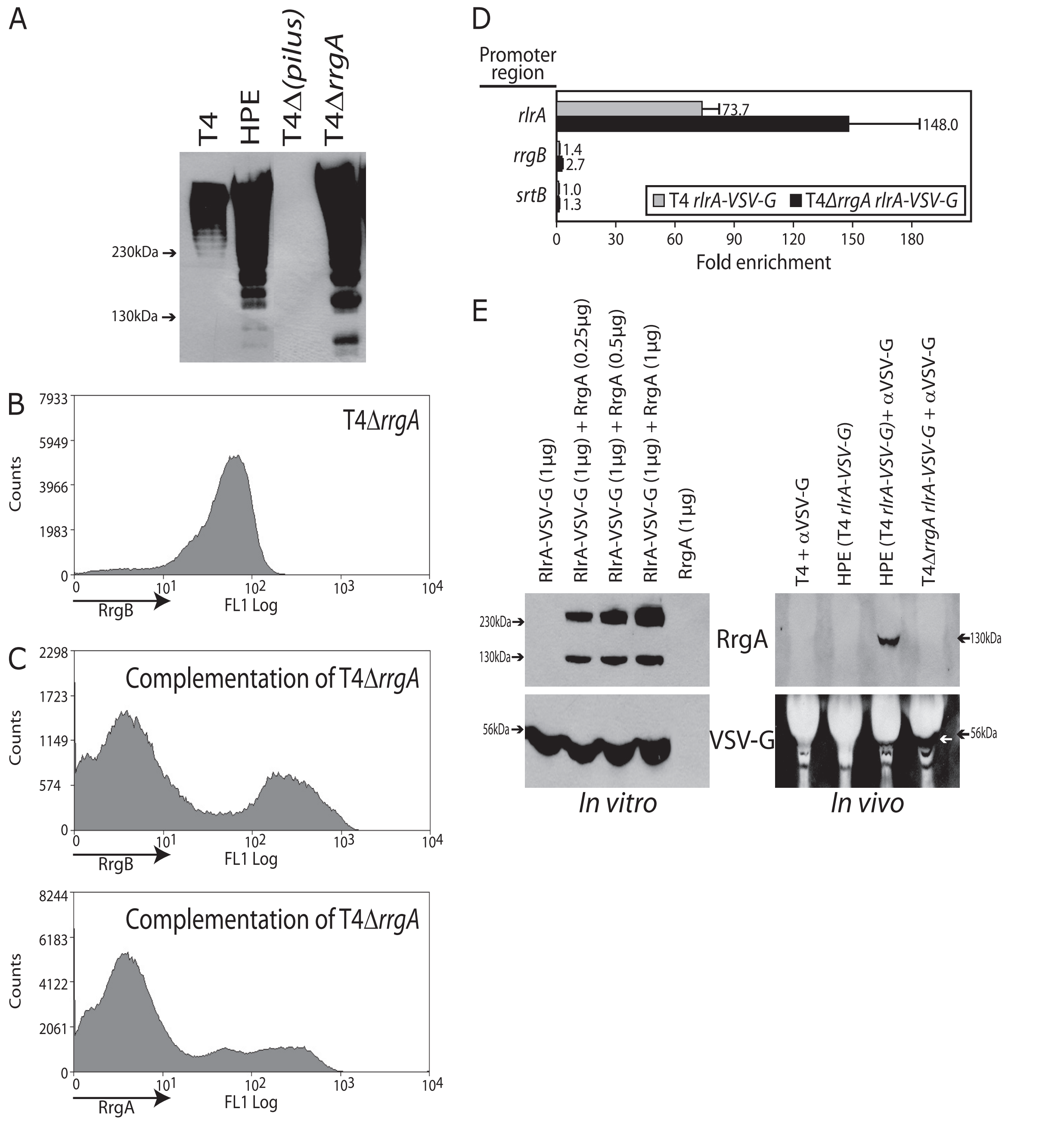

FIG. 4. The RrgA protein is a negative regulator of pilus expression that interacts directly with RlrA. (A) Detection of RrgB by Western blotting in T4, HPE, T4 Δ(pilus), and T4 Δ*rrgA* cells. The numbers of bacteria were equivalent in each lane. (B) Cells of the T4 Δ*rrgA* strain display only the HPE phenotype (20 strains evaluated, all strains HPE, and representative results shown). (C) Complementation of the T4 Δ*rrgA* mutant strain restores bistable expression of the pilus. (D) A ChIP experiment was performed measuring the association of RlrA with different promoters of the *rlrA* pathogenicity islet in strains T4 *rlrA*-VSV-G and T4 Δ*rrgA rlrA*-VSV-G. More RlrA was found associated with the *rlrA* promoter in T4 Δ*rrgA rlrA*-VSV-G cells than in T4 *rlrA*-VSV-G cells. RlrA did not detectably associate with the *srtB* promoter. (E) RlrA-VSV-G and RrgA proteins coprecipitate. (Left panel) Various amounts of RlrA-VSV-G and RrgA protein were incubated together, after which RlrA-VSV-G was immunoprecipitated with anti-VSV-G antibodies. There is a dose-dependent increase in detectable RrgA by Western blotting; no RrgA is detected in the absence of RlrA-VSV-G. The two bands corresponding to RrgA are also seen when the purified RrgA protein alone is run on a denaturing SDS polyacrylamide gel. (Right panel) To examine whether the two proteins interact when expressed by pneumococci, lysates of pneumococcal strains with or without a VSV-G-tagged version of RlrA and with or without RrgA were immunoprecipitated using anti-VSV-G (αVSV-G) antibodies. No RrgA protein is immunoprecipitated in the T4 strain in the absence of the VSV-G-tagged RlrA or in a T4 Δ*rgA rlrA*-VSV-G strain. In contrast, both proteins are readily detected by Western blotting when lysates from a strain that expresses the VSV-G-tagged RlrA and wild-type RrgA are used, confirming that the two proteins interact.

scribed here represents the first example of a bistable system in *S. pneumoniae.*

We show here that type 1 pilus expression in pneumococcus is tightly regulated by a system that results in only a minority (about 30%) of cells within a population expressing the pilus. This bistable expression of the pilus appears to be the consequence of the molecular interaction between RlrA, a positive regulator of pilus gene expression, and RrgA, a negative regulator of pilus gene expression. As we show here, in a strain in which RlrA expression is placed under the control of a heterologous, maltose-inducible promoter, the gradual addition of maltose results in a graded or monophasic increase in pilus expression, implicating the endogenous *rlrA* promoter in the bistable phenotype. Similarly, in the absence of RrgA, virtually all of the cells within a population display an HPE phenotype. We also show here that the two proteins RlrA and RrgA interact in a specific manner. This interaction appears to interfere, either directly or indirectly, with the binding of RlrA to its own promoter. Taken together, these data strongly suggest that the bistable phenotype is the result of the molecular interaction between a positive regulator and negative regulator of the pilus. We imagine that another important determinant of pilus bistability is positive autoregulation of the *rlrA* gene (15). This is consistent with our finding that the native *rlrA* promoter is required for bistable expression of the pilus. Indeed, at least one bistable switch in a pathogenic bacterium has been shown to be mediated by a positive feedback loop involving a transcription regulator (37). However, positive regulation of the *rlrA* gene by RrgA alone evidently does not suffice to mediate bistability, but instead requires input from the negative regulator RrgA.

Pili in pneumococcus have been implicated in a number of biological processes, including increased host cell adherence, biofilm formation, and inflammatory responses. It is plausible that these responses may, under some circumstances, either enhance or diminish the fitness of the organism. In the case of pneumolysin, the cholesterol-dependent cytolysin of pneumococcus, the presence of the toxin is associated with enhanced virulence in animal models, but the interaction of the toxin with Toll-like receptor 4 (TLR4) results in a reduced capacity to colonize the nasopharynx and cause disease in mice (26, 35). A similar situation may be occurring with the pilus, whereby the presence of the adherence-promoting RrgA protein may initially increase the ability of the organism to colonize, but may also interfere with long-term colonization by triggering inflammatory or acquired immune responses in the host.

Given the findings presented here, the role of pilus proteins in pathogenesis and their potential as vaccine candidates must be reexamined. We had previously reported that the presence of the pilus (at the DNA level) in pneumococcal isolates in children was not associated with increased virulence (5). Given the bistable nature of pilus expression, it is now apparent that studies of pilus expression in the context of clinical infection would be necessary to evaluate more fully the role of this structure in virulence. Similarly, while protection against invasive disease was demonstrated when pilus antigens were used as vaccines in mouse models, the bistability of pilus expression could conceivably interfere with such a strategy, since the pilus is not required for virulence in humans (5). Further experiments using the two phenotypic populations among piliated strains in animal models are planned and may allow for a more complete understanding of the role of the pilus at different stages of pneumococcal pathogenesis and immunity to this respiratory pathogen.

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