Journal of Bacteriology

An Epigenetic Switch Mediates Bistable Expression of the Type 1 Pilus Genes in Streptococcus pneumoniae

Alan Basset, Keith H. Turner, Elizabeth Boush, Sabina Sayeed, Simon L. Dove and Richard Malley

J. Bacteriol. **2012, 194(5):1088. DOI: 10.1128/JB.06078-11.**
Published Ahead of Print 22 December 2011.

JB
Journals.ASM.org

An Epigenetic Switch Mediates Bistable Expression of the Type 1 Pilus Genes in *Streptococcus pneumoniae*

Alan Basset, Keith H. Turner, Elizabeth Boush, Sabina Sayeed, Simon L. Dove, and Richard Malley

Division of Infectious Diseases, Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA

Expression of the pneumococcal type 1 pilus is bistable and positively regulated by the transcription factor RlrA. RlrA is also known to positively control its own expression. Here we present evidence that bistable expression of the type 1 pilus is mediated by the positive-feedback loop controlling *rlrA* expression.

Based on a screen designed to identify pneumococcal virulence genes in a mouse model of infection, the type 1 pilus was identified to be an important virulence factor (6). Further studies in mice have confirmed these findings, as the type 1 pilus was subsequently demonstrated to be a potent inflammatory agonist (1) and an adhesin (11) and to participate in biofilm formation (10). The structural proteins of the pilus have been evaluated in mice as vaccine candidates (5). At the same time, the role of the pneumococcal type 1 pilus in pneumococcal pathogenesis in humans remains uncertain, with epidemiological data conflicting. We have previously demonstrated that the prevalence of pilus genes was similar in strains isolated from blood or the nasopharynx of children; this lack of enrichment of piliated strains in bacteremic isolates strongly argues against a major role of the pilus in invasive disease (2). After the introduction of the pneumococcal conjugate vaccine Prevnar in 2000 in the United States, the prevalence of strains carrying the pilus genes declined dramatically, as a result of the association between the type 1 pilus and the capsular serotypes covered by the vaccine (2). However, several years later, with the emergence of replacement strains not covered by the vaccine, the prevalence of strains carrying the type 1 pilus genes returned to pre-2000 levels, arguing instead that the presence of these genes may confer an advantage to the organism (13).

Complicating the picture further, we and others recently demonstrated that type 1 pilus gene expression is bistable (3, 4). In particular, using flow cytometric analysis with antibodies to structural proteins of the pilus, we identified the existence of two different cell types within a clonal population of *Streptococcus pneumoniae* strain TIGR4 (T4). One of these cell types expressed the pilus (high pilus expression [HPE]), whereas the other did not (low pilus expression [LPE]). The same phenomenon was observed in cell populations of several other clinical pneumococcal strains (3). We were able to show that the bistable phenotype is dependent on the presence of the endogenous *rlrA* promoter and that bistable pilus expression is negatively regulated by RrgA, a structural component of the pilus. Our previous work, however, did not address the mechanistic basis for bistable pilus gene expression. Several mechanisms for the generation of phenotypically distinct subpopulations in a clonal population of bacteria have been described. For instance, phase-variable gene expression can arise from programmed stochastic modifications to DNA sequence or chemistry (reviewed in reference 19). In our case, we were unable to detect any changes to the *rlrA* promoter sequence between HPE and LPE populations (3), suggesting that changes to DNA sequence are not involved in controlling the observed phenotypic variability in the pneumococcal pilus.

Gene expression systems that involve transcription feedback can also give rise to phenotypic variability. For example, bistability can be mediated by a transcription activator acting cooperatively to positively regulate its own expression; bistability can occur due to the accumulation of the regulator, in a stochastically determined subset of cells, to a concentration above the threshold required to activate expression of the gene encoding the regulator. Such feedback-mediated bistability controls phenotypic variation in a number of bacteria, including the opportunistic pathogen *Pseudomonas aeruginosa* (18). We have previously shown that expression of *rlrA* from its endogenous promoter is required for bistable pilus expression. Furthermore, RlrA is known to regulate expression from its own promoter, creating a potential positive-feedback loop (7). We therefore hypothesized that bistable expression of the type 1 pilus genes is mediated by this positive-feedback loop, with cells in which *rlrA* expression is autoactivated displaying high intracellular concentrations of RlrA with concomitant high-level expression of the pilus genes.

A hallmark of feedback-mediated bistable systems is that they display a capacity for hysteresis (12). That is, the expression state of these systems appears to display a memory of previous expression states. This phenomenon has been observed in bistable expression of the *lac* operon in *Escherichia coli*, cell cycle progression in *Xenopus laevis* oocytes, and numerous synthetic gene expression systems involving positive feedback (reviewed in reference 9). Therefore, if the type 1 pilus genes of *S. pneumoniae* exhibit bistable expression due to a feedback-mediated bistable switch in *rlrA* expression, strains lacking this positive-feedback loop should no longer exhibit a hysteretic response to previous expression states. To test this prediction, we constructed a pair of strains that contain an inducible construct for ectopic expression of *rlrA* and either are wild type for *rlrA* at its native locus (and thus contain an intact positive-feedback loop) or contain a deletion of *rlrA* (and thus lack a positive-feedback loop). If *rlrA* comprises a feedback-

Received 26 August 2011 **Accepted** 16 December 2011
Published ahead of print 22 December 2011
Address correspondence to Richard Malley, richard.malley@childrens.harvard.edu.
S. L. Dove and R. Malley contributed equally to this article.

doi:10.1128/JB.06078-11

mediated bistable switch, then only the strain in which the positive-feedback loop is intact should maintain high expression of the RlrA-regulated type 1 pilus after ectopic *rlrA* expression is withdrawn. Using this experimental approach, we show here that positive feedback of *rlrA* is required for persistence of the HPE phenotype, suggesting that bistable expression of the type 1 pilus genes is mediated by the positive-feedback loop that involves RlrA.

In the studies described below, overlapping PCR products were generated and used to transform strains in which the bicistronic Janus cassette (17) replaced the gene of interest. Two different derivatives of TIGR4 (T4) were used in this study, one, called the "minus-feedback" strain (T4 *Pmal23-rlrA* Δ*rlrA*), in which the endogenous *rlrA* gene was deleted and reinserted elsewhere in the genome under the control of a modified maltose promoter (Fig. 1A), and another one, called the "plus-feedback" strain (T4 *Pmal23-rlrA*), which carries both the positively autoregulated endogenous *rlrA* gene and an exogenous copy of the *rlrA* gene under the control of the same modified maltose promoter (Fig. 1C). Both strains were grown for a period of 24 h, harvested for evaluation by flow cytometry, and then reinoculated at a low optical density daily under the indicated conditions over a period of 3 days. The flow cytometry procedure targeting the RrgB proteins of the pilus was performed as described previously (3). These strains were grown in Dulbecco modified Eagle medium (DMEM; Cellgro, Mediatech, Inc.) supplemented with either 1% glucose to repress expression from the maltose promoter (20) or 1% maltose to induce expression from the maltose promoter.

The results obtained for the minus-feedback strain are shown in Fig. 1B. On day 1, the majority of cells in the population exhibit mainly the LPE phenotype, with a minority of cells exhibiting the HPE phenotype. The latter population is likely a consequence of residual expression from the modified maltose promoter. The relative composition of the population of cells obtained after successive growth of cells from day 1 in DMEM supplemented with glucose remains similar over days 2 and 3. The degree to which the pilus is expressed in cells in these populations indicates that in the presence of glucose, exogenous *rlrA* expression is repressed to a level comparable to that of endogenous *rlrA* expression in cells of wild-type *S. pneumoniae* in the LPE state. When cells from day 1 were grown in DMEM supplemented with maltose, a majority of cells in the population shifted from the LPE to HPE states, indicating successful exogenous expression of the *rlrA* gene. Importantly, these cells exhibit a degree of pilus expression similar to that observed in cells of wild-type *S. pneumoniae* in the HPE state, indicating that the *rlrA* gene can be expressed from the maltose promoter under induction to a degree similar to that exhibited by expression from its native locus. When cells from day 2 grown in maltose were regrown on the following day (day 3) in DMEM supplemented with maltose, cells remained in the HPE state, suggesting that pilus expression can be maintained over that time period under ectopic *rlrA* expression. In contrast, when the same cells from day 2 grown in the presence of maltose were regrown in DMEM supplemented with glucose, cells in the HPE state reverted to the LPE state, consistent with repression of ectopic *rlrA* gene expression in the presence of glucose. The results from this strain, which carries a single copy of *rlrA* under the control of the inducible maltose promoter, suggest that pilus gene expression is transient after brief ectopic expression of *rlrA* in the absence of positive feedback at the *rlrA* locus.

We then conducted the same experiment with cells of the plus-feedback strain. The pilus is bistably expressed on day 1 when cells are grown in DMEM supplemented with glucose, and cells exhibit both the LPE and HPE phenotypes (Fig. 1D). The relative composition of the population of cells obtained after successive growth of cells from day 1 in DMEM supplemented with glucose remains similar over days 2 and 3. On day 2, when grown in DMEM supplemented with maltose, the entire population of cells switches to the HPE phenotype in a manner similar to that observed with the minus-feedback strain (Fig. 1D and B). When the cells of the plus-feedback strain are subsequently cultured in DMEM supplemented with glucose on day 3 (i.e., under conditions in which ectopic expression of *rlrA* is repressed), cells remain in the HPE state (Fig. 1D). These results suggest that expression of the type 1 pilus exhibits hysteresis when the positive-feedback loop is intact. This is the first demonstration of hysteresis in bistable expression of the pneumococcal type 1 pilus and supports the hypothesis that positive feedback of *rlrA* mediates bistable pilus gene expression.

These data therefore provide a plausible explanation for the stability of the HPE state: once a certain threshold concentration of RlrA sufficient to activate the positive-feedback loop is reached, autoactivation maintains high-level expression of *rlrA* with concomitant high-level expression of the type 1 pilus genes. It should be noted that our experiments do not specifically address the mechanism by which RlrA positively regulates expression of its own gene. RlrA may function as an activator simply by contacting RNA polymerase; alternatively, it is formally possible that RlrA functions through a more complex mechanism and exerts its effects by influencing the methylation state of the *rlrA* promoter (19).

Given our findings, one could of course wonder why only a fraction of cells expresses the pilus at any one time. However, as we observed (3), there is a demonstrable rate of reversion of HPE cells to the LPE state, and the balance between these rates presumably determines the proportion of cells in each expression state. In relation to this, we have shown previously that RrgA interacts directly with RlrA and negatively regulates pilus gene expression (3). Moreover, others have demonstrated the importance of other regulators in the control of pilus gene expression (8, 15, 16). Thus, the complex interplay between these negative regulators, including RrgA, and the positive regulator RlrA may determine this equilibrium and potentially provide a mechanism for reversion to the LPE phenotype. Interestingly, both RrgA and RlrA are encoded on the *rlrA* pilus pathogenicity island, suggesting that acquisition of this island conferred not only the genes encoding the pilus but also the capacity for its bistable expression. This has important implications for the spread of the type 1 pilus genes among pneumococcal strains in the clinical setting, as well as for horizontal gene transfer more broadly.

It is thus apparent that *S. pneumoniae* maintains very tight control of type 1 pilus gene expression. The type 1 pilus genes are present in only a minority (25%) of strains, and, on the basis of circumstantial epidemiological data, may be more common in immunologically naïve individuals than those with preexisting antibodies (2, 14). Despite near eradication of strains that contain the pilus genes soon after the introduction of the pneumococcal conjugate vaccine, strains that contain the pilus genes have since returned to previous levels, which argues for a relative advantage of these genes under certain conditions. However, the fact that the pilus is bistably expressed, a phenomenon that we propose arises

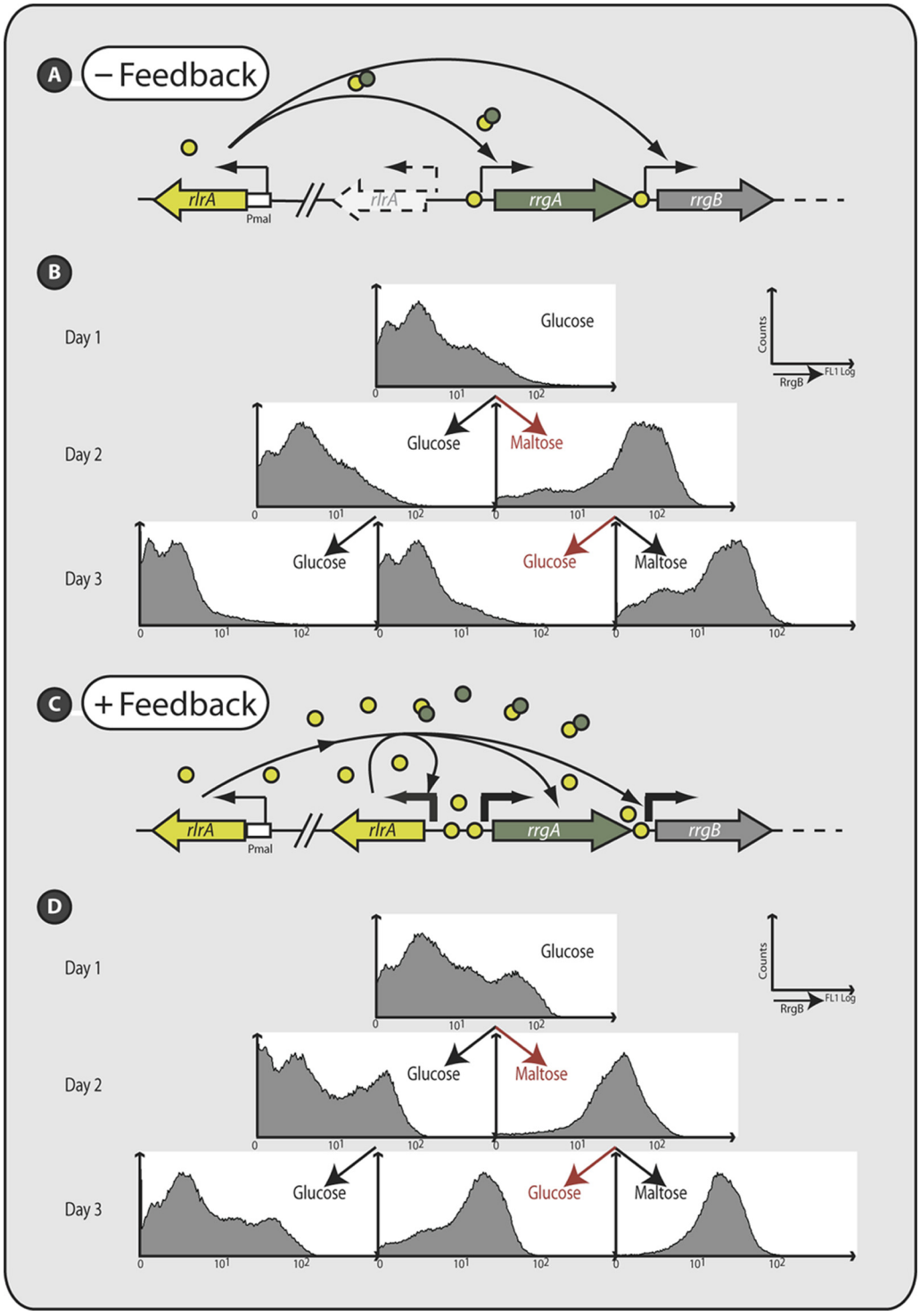

FIG 1 An RlrA-mediated positive-feedback loop mediates bistable expression of the pneumococcal type 1 pilus genes. (A and C) Diagrams of the minus-feedback (A) and plus-feedback (C) strains used in this study. The dashed outline of the *rlrA* gene represents a deletion of this gene. Light circles represent the RlrA protein, and dark circles represent the RrgA protein. (B and D) The minus-feedback (B) and plus-feedback (D) strains were grown for 3 days successively. Each day, cells from the previous day were grown in medium containing either maltose or glucose, for induction or repression, respectively, of ectopic *rlrA* expression from the modified maltose promoter. Each plot is a histogram of fluorescence intensity obtained after analyzing a population of cells stained with antibodies directed against the major pilus subunit protein RrgB. The red arrows indicate a comparison emphasized in the text, namely, growth of the cells before, during, and after a brief pulse of ectopic *rlrA* expression.

due to positive feedback of the positive regulator RlrA, further supports the view that a complex balance of benefits and costs to the expression of pilus genes must exist. These may depend on factors such as age, preexisting immunity, and site of infection or colonization. Overall, our findings underscore the need for thorough investigations of the pattern and kinetics of pilus expression during infection in animal models.

ACKNOWLEDGMENTS

We thank Michael Wessels and Porter Anderson (Children's Hospital Boston) for helpful discussions and suggestions.

R.M. gratefully acknowledges support from the Translational Research Program at Children's Hospital Boston. This work was supported by National Institutes of Health grants AI069007 (to S.L.D.) and AI066013 (to R.M.).

REFERENCES

1. **Barocchi MA, et al.** 2006. A pneumococcal pilus influences virulence and host inflammatory responses. Proc. Natl. Acad. Sci. U. S. A. **103**:2857–2862.
2. **Basset A, et al.** 2007. Association of the pneumococcal pilus with certain capsular serotypes but not with increased virulence. J. Clin. Microbiol. **45**:1684–1689.
3. **Basset A, et al.** 2011. Expression of the type 1 pneumococcal pilus is bistable and negatively regulated by the structural component RrgA. Infect. Immun. **79**:2974–2983.
4. **De Angelis G, et al.** 2011. The *Streptococcus pneumoniae* pilus-1 displays a biphasic expression pattern. PLoS One **6**:e21269.
5. **Gianfaldoni C, et al.** 2007. *Streptococcus pneumoniae* pilus subunits protect mice against lethal challenge. Infect. Immun. **75**:1059–1062.
6. **Hava DL, Camilli A.** 2002. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. Mol. Microbiol. **45**:1389–1406.
7. **Hava DL, Hemsley CJ, Camilli A.** 2003. Transcriptional regulation in the *Streptococcus pneumoniae rlrA* pathogenicity islet by RlrA. J. Bacteriol. **185**:413–421.
8. **Hemsley C, Joyce E, Hava DL, Kawale A, Camilli A.** 2003. MgrA, an orthologue of Mga, acts as a transcriptional repressor of the genes within the *rlrA* pathogenicity islet in *Streptococcus pneumoniae.* J. Bacteriol. **185**:6640–6647.
9. **Mitrophanov AY, Groisman EA.** 2008. Positive feedback in cellular control systems. Bioessays **30**:542–555.
10. **Munoz-Elias EJ, Marcano J, Camilli A.** 2008. Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. Infect. Immun. **76**:5049–5061.
11. **Nelson AL, et al.** 2007. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae.* Mol. Microbiol. **66**:329–340.
12. **Ninfa AJ, Mayo AE.** 2004. Hysteresis vs. graded responses: the connections make all the difference. Sci. STKE **2004**:pe20.
13. **Regev-Yochay G, et al.** 2010. Re-emergence of the type 1 pilus among *Streptococcus pneumoniae* isolates in Massachusetts, USA. Vaccine **28**:4842–4846.
14. **Regev-Yochay G, et al.** 2009. The pneumococcal pilus predicts the absence of *Staphylococcus aureus* co-colonization in pneumococcal carriers. Clin. Infect. Dis. **48**:760–763.
15. **Rosch JW, Mann B, Thornton J, Sublett J, Tuomanen E.** 2008. Convergence of regulatory networks on the pilus locus of *Streptococcus pneumoniae.* Infect. Immun. **76**:3187–3196.
16. **Song XM, Connor W, Hokamp K, Babiuk LA, Potter AA.** 2009. The growth phase-dependent regulation of the pilus locus genes by two-component system TCS08 in *Streptococcus pneumoniae.* Microb. Pathog. **46**:28–35.
17. **Sung CK, Li H, Claverys JP, Morrison DA.** 2001. An *rpsL* cassette, janus, for gene replacement through negative selection in *Streptococcus pneumoniae.* Appl. Environ. Microbiol. **67**:5190–5196.
18. **Turner KH, Vallet-Gely I, Dove SL.** 2009. Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator. PLoS Genet. **5**:e1000779.
19. **van der Woude MW, Baumler AJ.** 2004. Phase and antigenic variation in bacteria. Clin. Microbiol. Rev. **17**:581–611.
20. **van Wezel GP, White J, Young P, Postma PW, Bibb MJ.** 1997. Substrate induction and glucose repression of maltose utilization by *Streptomyces coelicolor* A3(2) is controlled by *malR*, a member of the *lacI-galR* family of regulatory genes. Mol. Microbiol. **23**:537–549.