

Loss of both Holliday junction processing pathways is synthetically lethal in the presence of gonococcal pilin antigenic variation

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Summary

The obligate human pathogen *Neisseria gonorrhoeae* (Gc) has co-opted conserved recombination pathways to achieve immune evasion by way of antigenic variation (Av). We show that both the RuvABC and RecG Holliday junction (HJ) processing pathways are required for recombinational repair, each can act during genetic transfer, and both are required for pilin Av. Analysis of double mutants shows that either the RecG or RuvAB HJ processing pathway must be functional for normal growth of Gc when RecA is expressed. HJ processing-deficient survivors of RecA expression are enriched for non-piliated bacteria that carry large deletions of the *pilE* gene. Mutations that prevent pilin variation such as *recO*, *recQ*, and a *cis*-acting *pilE* transposon insertion all rescue the RecA-dependent growth inhibition of a HJ processing-deficient strain. These results show that pilin Av produces a recombination intermediate that must be processed by either one of the HJ pathways to retain viability, but requires both HJ processing pathways to yield pilin variants. The need for diversity generation through frequent recombination reactions creates a situation where the HJ processing machinery is essential for growth and presents a possible target for novel antimicrobials against gonorrhoea.

Introduction

Microbial pathogens encounter many different barriers that must be overcome to successfully establish infection. The innate and adaptive immune systems provide a mul-

tilayered array of defences that successful pathogens must contend with. Opportunistic pathogens often require some level of immune dysfunction to establish infection. In contrast, professional pathogens possess one or more strategies to overcome immune surveillance. *Neisseria gonorrhoeae* (Gc) is the sole aetiologic agent of the sexually transmitted disease gonorrhoea. Gonorrhoea has been evident within humans for all recorded history (Wain, 1947; Morton, 1977; Rothenberg, 1993) and has evolved to specifically thrive within the human genital tract. However, even without antibiotic treatment, gonorrhoea is usually self-limiting. Gc relies upon sexual networks to spread and persist within infected populations. Consistent with this continual movement between individuals is the observation that high-risk people can contract gonorrhoea countless times and never demonstrate immunity to the disease. One reason for the lack of effective immunity to reinfection is the capacity of Gc to antigenically vary its surface at high frequency. The surface exposed Opa proteins and the lipooligosaccharide both undergo high-frequency antigenic variation (Av) by altering nucleotide repeat tracts (Kline *et al.*, 2003). In contrast, the pilus undergoes high-frequency Av by homologous recombination reactions between one of many silent pilin loci and a single expressed pilin gene (Kline *et al.*, 2003).

Pilin variation is mediated by the homologous recombination machinery and is dependent on RecA and RecX, the RecF-like recombination pathway, the chromosome organizing factor RdgC, and the Rep helicase (Seifert, 1997; Mehr and Seifert, 1998; Stohl and Seifert, 2001; Skaar *et al.*, 2002; Kline and Seifert, 2005a). These conserved recombination proteins help mediate high-frequency gene conversion reactions that transfer short stretches of variant sequences bordered by small segments of sequence identity between silent pilin loci and the expressed gene (Howell-Adams and Seifert, 2000; Criss *et al.*, 2005). These homologous recombination processes also contribute to survival from DNA damaging agents and some to DNA transformation competence (Kline *et al.*, 2003). A genetic screen for factors interfering with pilin variation revealed a number of genes that, when mutated, reduced the frequency of pilin variation. Two pilin Av-deficient mutants isolated were inactivated for the Holliday junction (HJ) processing helicases RuvAB (*ruvA* inactivation) and

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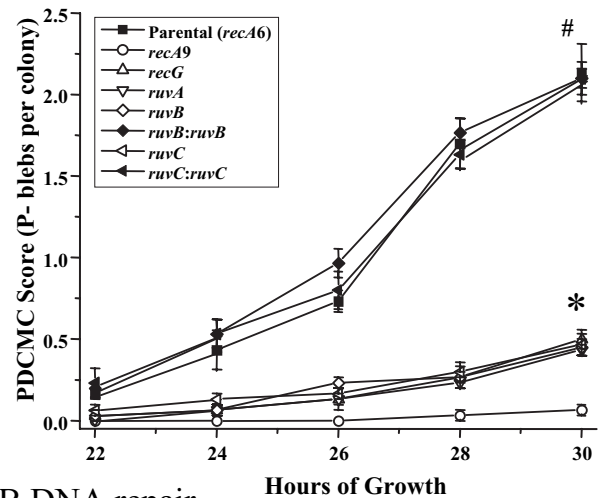
RecG (*recG* inactivation) (Sechman *et al.*, 2005). In addition, a transposon insertion in the non-coding region upstream of the *pilE* expression locus also disrupted pilin Av, apparently acting *in cis* (Sechman *et al.*, 2005). Here we show that a *ruvB* and a *ruvC* mutant each is deficient for pilin Av. Surprisingly, when any of the three *ruv* mutations were combined with a *recG* mutation there was a severe RecA-dependent growth deficiency for each mutant strain. This growth deficiency could be overcome by a variety of *cis*- and *trans*-acting mutations that each disrupt pilin Av, proving that the process of pilin Av specifically makes the *recG* and *ruv* mutations synthetically lethal. We propose that pilin Av intermediates contain HJs and the inability to process HJs creates a structure that blocks chromosomal replication and results in a growth deficiency.

Results

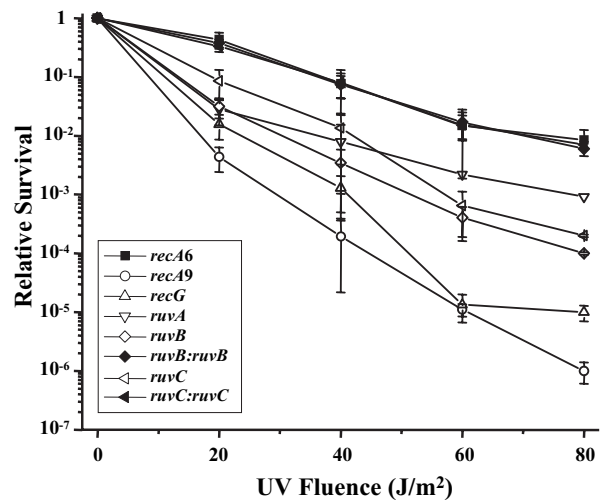
Holliday junction processing is involved in pilin variation, DNA repair and DNA transformation

A genetic screen previously revealed 30 genes that showed reduced levels of pilin variation when mutated, including the Gc *recG* and *ruvA* genes (Sechman *et al.*, 2005). In bacteria there are two major helicases involved in the processing of HJs to promote branch migration and the formation of heteroduplexes, RuvAB and RecG. DNA duplexes joined by HJs and processed by RuvAB are subsequently separated by the action of the dedicated endonuclease RuvC (Sharples *et al.*, 1999). Analysis of the sequenced gonococcal FA1090 genome revealed that genes encoding proteins similar to both RuvB and RuvC are also present in Gc (Genebank Accession: AE004969). Mutants carrying a loss-of-function mutation of either of these genes also displayed reduced pilin Av to levels similar to the *ruvA* and *recG* mutants, confirming that both pathways are required for pilin variation (Fig. 1A). The pilin Av deficiency could be complemented by supplying an expressed copy of the *ruvB* or *ruvC* genes in the mutant strains from an ectopic locus (Fig. 1A). The three *ruv* mutants and the *recG* mutant all showed reduced survival when exposed to UV light supporting the hypothesis that HJ processing is important for DNA repair (Fig. 1B) (Mehr and Seifert, 1998). The UV survival phenotypes were complemented by ectopic expression of a function gene in the *ruvB* and *ruvC* backgrounds (Fig. 1B). We had previously reported that the *ruvA* and *recG* mutants were not affected for DNA transformation competence (Sechman *et al.*, 2005). Since that report, it was found that a *Neisseria meningitidis* *recG* mutant showed reduced transformation competence (Sun *et al.*, 2005). We therefore retested the DNA transformation competence of the HJ processing mutants. In contrast to the previous assays, in which DNA exposure to the bacteria

A Pilin variation



B DNA repair



C DNA transformation

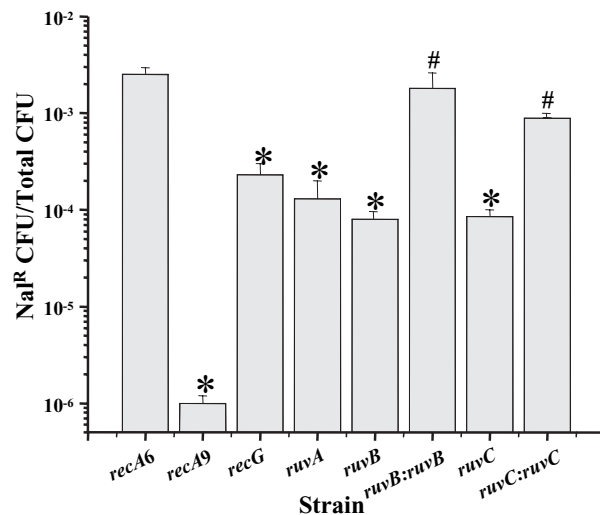


Fig. 1. Effect of HJ processing mutants on pilin Av, DNA repair and DNA transformation. Parental strain is FA1090 expressing the 1-81-S2 pilin variant isolated from a human volunteer infection (Seifert *et al.*, 1994) carrying the *recA6* allele. This strain is phenotypically RecA⁻ without IPTG in the growth medium and RecA⁺ Gc when grown in the presence of IPTG (Seifert, 1997). All strains are isogenic except for the indicated loss-of-function mutation and where appropriate the wild-type gene expressed from an irrelevant locus.

A. Pilin variation was measured using the PDCMC assay as previously described (Sechman *et al.*, 2005). Approximately 10 P⁺ colonies were assayed and the number of non-piliated outgrowths recorded for each time indicated.

B. UV survival was measured by exposing dilutions of the indicated strains to the indicated UV dose. Each value is reported relative to the unirradiated control. The survival curves for the parental strain and the complemented *ruvB:ruvB* and *ruvC:ruvC* cultures are indistinguishable.

C. DNA transformation competence was tested using the pSY6 plasmid which carries a *gyrB* allele that confers resistance to nalidixic acid when recombined into the chromosome (Stein *et al.*, 1991).

Error bars represent the standard error of the mean of at least three experiments. * indicates statistical difference from *recA6* at $P \leq 0.05$. # indicates statistical difference from *ruvB* and *ruvC* at $P \leq 0.05$.

was continuous (Sechman *et al.*, 2005), the time the bacteria were exposed to transforming DNA was limited to 15 min with the addition of DNase I. By this method, all of the HJ processing mutants showed a significant reduction in transformation competence and this phenotype was complemented by the functional gene (Fig. 1C). We conclude that both the RecG and RuvABC HJ processing pathways contribute to pilin variation, DNA repair and DNA transformation.

Holliday junction processing is necessary for survival when RecA is expressed

Each individual HJ processing mutant retained measurable abilities to conduct pilin Av, DNA repair and DNA transformation relative to the *recA* mutant (Fig. 1). We therefore constructed double mutant strains deficient in both HJ processing pathways (*recG* and *ruvA*, *ruvB* or *ruvC*) to determine whether a HJ processing-deficient strain would be similar to a *recA* mutant. Surprisingly, the HJ double mutants all showed a severe growth defect when RecA was expressed (Fig. 2B and C). In the absence of RecA expression, the growth of HJ double mutants was similar to the single mutants and to the

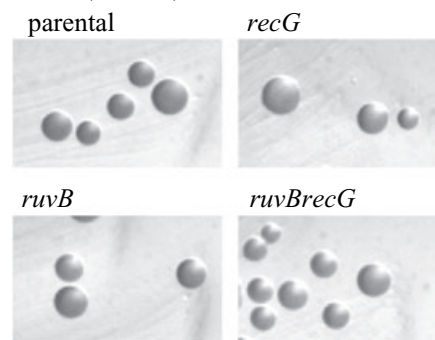
Fig. 2. Effect of mutations in HJ processing on RecA-dependent growth and pilin variation. Strains are the same as in Fig. 1 and were grown for 24 h.

A. Colonies of HJ processing mutants without IPTG (RecA⁻).

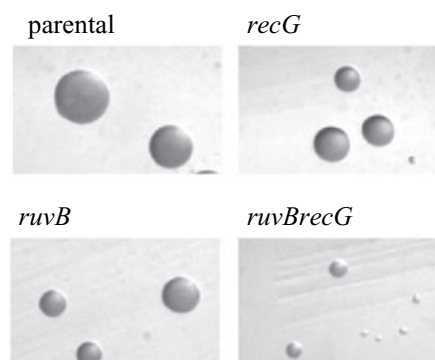
B. Colonies of HJ processing mutants with IPTG (RecA⁺).

C. Quantification of HJ processing mutant growth in colonies with IPTG (RecA⁺) or without IPTG (RecA⁻), relative to the parental strain.

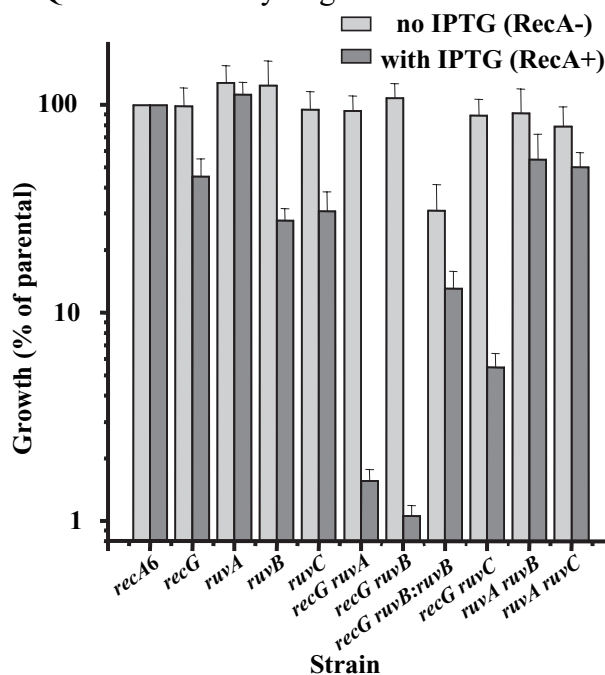
A No IPTG (RecA⁻)



B with IPTG (RecA⁺)



C Quantitative assay of growth



parental strain with functioning HJ processing systems (Fig. 2A and C). The growth defect of the *recG ruvB* double mutant could be complemented by an overexpressed copy of *ruvB* in an ectopic locus (Fig. 2C). As

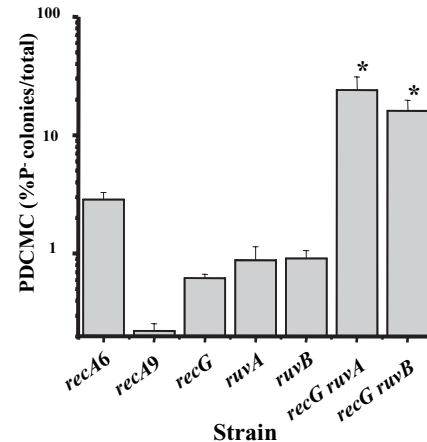
expected, there was no growth defect found with *ruvA* *ruvC* or *ruvB* *ruvC* double mutants, where only one HJ processing pathway was disrupted (Fig. 2C). These results strongly suggest that RecA-catalysed recombination results in a structure that must be acted on by one of the HJ processing systems, or growth becomes severely restricted. It was not clear from these data whether this growth deficiency was dependent on general recombination processes or was specific for a particular recombination pathway.

Pilin Av is responsible for the HJ processing requirement

The HJ processing single mutants were originally isolated as deficient in producing non-piliated variants (Sechman *et al.*, 2005), a phenotype shared by the additional HJ mutants created in this work (Fig. 3A). However, HJ double mutants surviving RecA induction were enriched for non-piliated variants (Fig. 3A). These HJ double mutant survivors of RecA expression were analysed to determine why the loss of HJ processing led to a RecA-dependent growth defect. The mechanism underlying this increased frequency of non-piliated variants in the HJ double mutant was revealed by a large increase in *pilE* deletions in the double mutant background (Fig. 3B). Interestingly, the non-piliated variants found after RecA expression in the HJ single mutants also showed increased *pilE* deletions, even though the overall frequency of non-piliated variants in the HJ processing single mutants was reduced (Fig. 3A). A HJ double mutant carrying a *pilE* deletion no longer showed a RecA-dependent growth deficiency, demonstrating that an intact *pilE* gene was required for the HJ processing-related growth deficiency. Furthermore, strains with an intact *pilE* and a non-piliated colony morphology retained the RecA-dependent growth defect (Fig. 4), demonstrating that loss of *pilE* and not the change in colony morphology alleviated the growth deficiency. The remainder of the surviving population was piliated and did not show high-frequency sequence changes at *pilE* (data not shown). It is likely that these surviving progeny represent bacteria that never initiated the process of pilin Av. We assume that the variability observed for the fraction of surviving HJ-deficient bacteria to RecA expression from experiment-to-experiment reflects when pilin Av was initiated and when a *pilE* deletion event occurred to present an escape mutant (Figs 2 and 3).

While the requirement for an intact *pilE* suggested that the growth deficiency was related to pilin variation, we tested whether mutations known to interfere with pilin Av would alter the growth defect. Introduction of a *recO*, *recJ* or *recQ* loss-of-function mutation into the HJ double mutant blocked the RecA-dependent growth defect (data

A Non-piliated Colonies



B *pilE* deletions

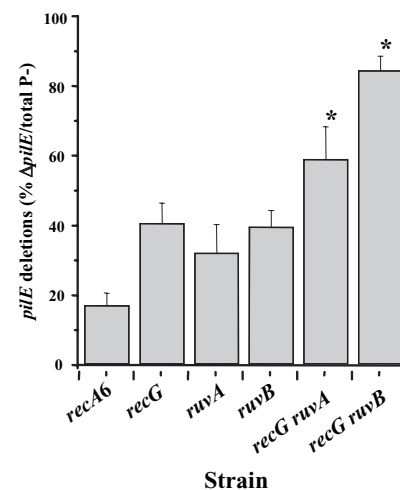


Fig. 3. Analysis of survivors of RecA induction.

A. Per cent of P⁻ colony variants after IPTG induction. Strains are the same as in Fig. 1 and were grown for 24 h in the presence of IPTG, collected and grown on solid medium without IPTG for 24 h before scoring for PDCMC under a stereomicroscope.

B. Per cent of P⁻ colony variants with a *pilE* deletion after IPTG induction. Random colonies from panel A which showed a non-piliated colony morphology were lysed and the presence of the *pilE* locus determined by PCR using the oligonucleotides PILSART and SP3A (Wright *et al.*, 1994). * indicates statistical difference from parental by Student's *t*-test with a *P* < 0.5.

not shown). These results show that a functional RecF-like recombination pathway is required for the growth defect and support the hypothesis that RecA acts in conjunction with the RecF-like recombination pathway to create HJs during pilin Av. However, as the RecF-like pathway participates in both pilin Av and DNA repair processes (Mehr and Seifert, 1998; Skaar *et al.*, 2002; Sechman *et al.*, 2005) we wanted to specifically ask whether the growth phenotype of the HJ processing mutant was directly linked to pilin Av. To this end, the effect of two transposons inserted immediately upstream of *pilE*

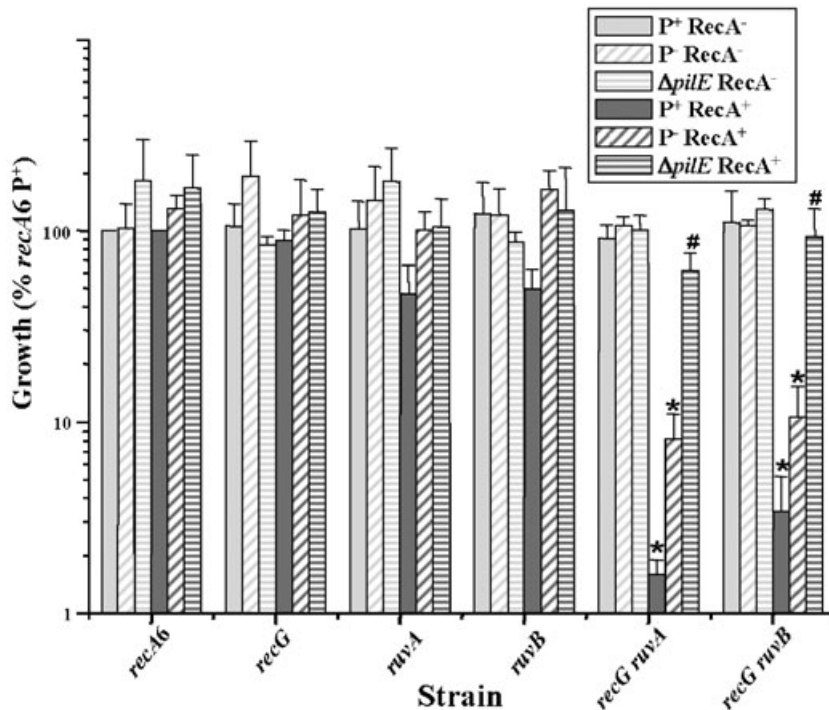


Fig. 4. Deletion of *pilE* in *recG ruvA* and *recG ruvB* mutant strains alleviates the RecA-dependent growth defect. All variants are FA1090 and contain the *recA6* allele. P⁺– indicates a variant with intact *pilE*, pilated colony morphology. P[–] represents a variant with an intact *pilE*, non-piliated colony morphology. ΔP represents a variant with a deleted *pilE*, non-piliated colony morphology. RecA[–] indicates that the strain was grown in the absence of IPTG, and RecA⁺ indicates that the strain was grown in the presence of IPTG. Growth of each strain was measured by determining cfu per colony after 24 h. Error bars represent the standard error of the mean of three experiments. * indicates a statistically significant difference from *recA6* P⁺. # indicates a statistically significant difference from *recG ruvA* and *recG ruvB* P⁺ RecA[–] and P[–] RecA⁺ at $P \leq 0.05$.

that differentially affect pilin Av were tested (Fig. 5A). One transposon insertion was previously shown to block pilin Av (*pilE::Tn5#1*) (Sechman *et al.*, 2005) to produce a non-varying colonial morphology (P^{nv}), and a second transposon insertion (*pilE::Tn5#9*) was found 83 bp upstream of the #1 transposon insertion that had no effect on pilin Av (Fig. 5A) (K.A. Kline, A. Criss, A. Wallace and H.S. Seifert, in preparation). Upon RecA induction in the HJ processing double mutant, the #1 transposon insertion prevented a growth defect, while the #9 transposon insertion retained the RecA-dependent growth defect (Fig. 5B). Neither transposon insertion altered the growth of the parental strains, are within or near an open reading frame, or alter *pilE* expression (Sechman *et al.*, 2005). These results conclusively show that pilin Av is directly responsible for the growth defect in the HJ processing mutants.

Discussion

Holliday junctions are central to all homologous recombination and many site-specific recombination systems (Lilley and White, 2001). Usually one HJ processing system is required for recombination, or different HJ processing systems can substitute for one another to facilitate recombination. The bacterial HJ processing pathways are also proposed to act on collapsed replication forks to allow RecF or RecBCD pathway recombinases to create a structure that can be acted on by the replication restart factors (Michel, 2000). As we have previously demonstrated that mutations in *priA*, the primary mediator of

replication restart, do not affect pilin Av (Kline and Seifert, 2005b), it is unlikely that the link between HJ processing and growth is related to replication restart. The requirement of both the Ruv and RecG systems for pilin Av frequencies similar to wild type suggests that these two separate HJ processing systems act at different steps during recombination or upon different substrates formed during the recombination process.

This is not the first instance where HJ processing has been demonstrated to be required for an organism's viability. While many bacterial species can tolerate having both the Ruv and RecG systems interrupted in the presence of an active *recA* gene with no reported growth defects (Asai and Kogoma, 1994; Beam *et al.*, 2002), there are two previous examples where a HJ processing deficiency caused problems. Attempts to isolate transformants of *Bacillus subtilis* that were deficient in both *ruvAB* and *recG*, *ruvAB* and *ruvU* (a *ruvC* homologue), or *ruvU* *recG* were unsuccessful for unknown reasons (Sanchez *et al.*, 2005). These mutations were not analysed with respect to RecA expression and it may have been the process of DNA transformation that made constructing the double mutants problematic. Moreover, the inability to isolate a *ruvAB*, *ruvU* double mutant in *B. subtilis* suggests there are both commonalities and differences between HJ processing within *B. subtilis* and *N. gonorrhoeae*. In addition, *E. coli* engineered to produce high-frequency double-stranded breaks through the action of the Scel endonuclease, *recG* and *ruvA* mutations were synthetically lethal (Meddows *et al.*, 2004). It is

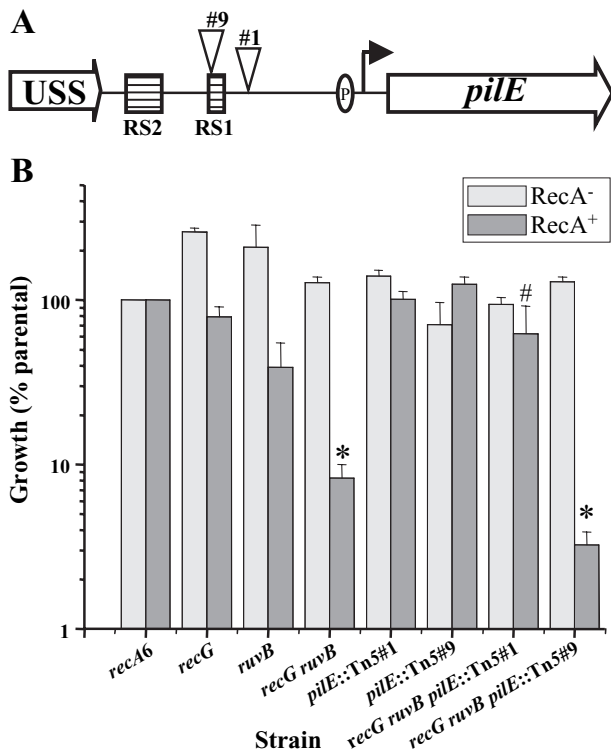


Fig. 5. A *cis*-acting transposon mutation rescues the HJ processing deficiency growth phenotype.

A. Map of the *pilE* upstream region showing the location of a transposon insertion that is deficient for pilin Av and produces a piliated non-varying colonial morphology (P^{ru}) (*pilE*::Tn5#1) or is wild type for pilin Av (*pilE*::Tn5#9). The distance between the sites of transposon insertions is 83 bp. The upstream silent pilin locus (USS), repeat sequence (RS1 and RS2), promoter (P) and transcriptional start (arrow) are shown.

B. The *pilE*::Tn5#9 and *pilE*::Tn5#1 transposon derived mutants differentially affect the RecA-dependent growth of the HJ processing mutants.

possible that the synthetic lethality of the *Scel* expressing *E. coli* and Gc undergoing pilin Av are the result of similar events (see below). Taken together, these data suggest that loss of HJ processing may only be a problem for bacterial cells when hetroduplex is formed with the bacterial chromosome at a high enough frequency to cause lethality due to the structures that are not resolved.

These results, along with the previous studies into the mechanisms of pilin Av, allow us to make predictions about the DNA recombination processes mediating pilin Av. We propose that an unknown initiator acting on the DNA upstream of *pilE* begins the process of pilin Av, and that the action of the initiator is blocked by the *pilE*::Tn5#1 transposon insertion (Fig. 6A). This proposition is based on the fact that the transposon insertion does not map within any open reading frame and does not affect *pilE* expression (Sechman *et al.*, 2005) and that it exerts its effect prior to the action of the recombination factors. The most likely activity for an initiator of recombination is an endonuclease

A Initiation event in Region 5' of *pilE*
(unknown effector)

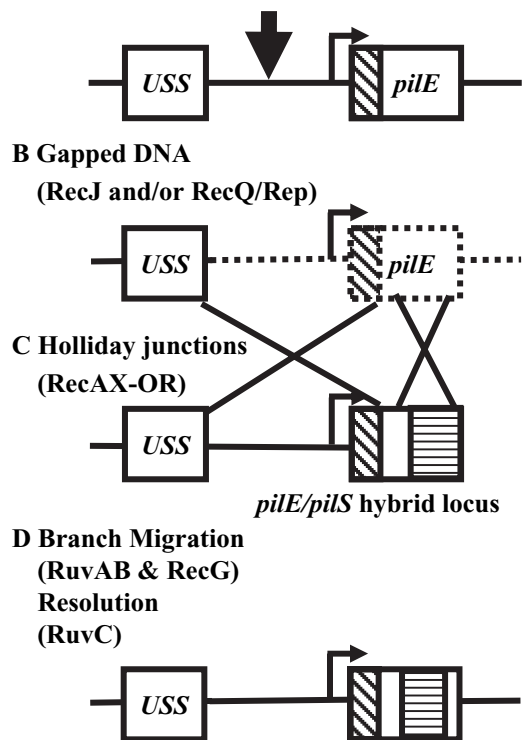


Fig. 6. A proposed pathway for recombination leading to pilin Av. A. Initiation event in region, possibly a single-stranded nick, defined by the *pilE*::Tn5#1 transposon insertion. B. Creation of a gapped substrate by the action of the exonuclease RecQ and/or the nucleases RecJ and Rep. C. Formation of one or two HJs by RecAX and RecOR. D. Branch migration by RuvAB and RecG followed by resolution by RuvC and possibly another undefined endonuclease for RecG.

which produces a double-stranded break or a single-stranded nick. As pilin Av depends on the RecF-like pathway (Mehr and Seifert, 1998), which acts on gapped substrates (Morimatsu and Kowalczykowski, 2003), the simplest model of initiation proposes a single-stranded nick, although an initiating double-stranded break cannot be ruled out. If a nick initiates, components of the RecF-like recombination pathway (i.e. RecJ and/or RecQ), or other enzymes such as Rep could process the *pilE* DNA into a gapped substrate, which would be the product of a single-stranded nick (Fig. 6B). RecA can be loaded onto this gapped substrate with the help of the RecOR complex (Morimatsu and Kowalczykowski, 2003). The RecX protein can also aid RecA to promote recombination with a homologous double-stranded molecule (Stohl and Seifert, 2001). The identity of this double-stranded substrate is presently unknown, but could either be the previously postulated hybrid intermediate which shares sequence identity with the *pilE* upstream region (Howell-Adams and

Seifert, 2000), or a silent locus sequence from a donor chromosome (not shown). This RecAXOR-mediated recombination event would form HJs, which would be acted upon by both the RuvAB and RecG helicases to promote branch migration and produce extended regions of heteroduplex (Fig. 6D). Alternatively, one of these HJ processing systems could be dedicated to producing the hybrid intermediate, while the other is responsible for processing the final HJs (Fig. 6C). We do not favour this hypothesis because inactivation of a HJ processing system that specifically alters hybrid intermediate formation would not be predicted to interfere with growth. Regardless of which of these possibilities is correct, both HJ processing systems are required for pilin Av, and in their absence, a HJ structure is formed that severely limits Gc growth. We have not determined the precise structure of this HJ-containing intermediate, but as there is more than one complete copy of the gonococcal chromosome within each monococcus (Tobiason and Seifert, 2006), it is intriguing to speculate that the lethal structure is a fusion between two chromosomes. We can only conclude from the data presented in this work that at least one HJ, linking two *pil*-containing DNA duplexes, forms a structure that inhibits growth.

In all cells, recombination processes can be stimulated to enable DNA damage repair and specialized programmes like meiosis, antibody diversification, mating type interconversion, or Av. The frequency of Gc pilin variation has been measured to be at least 4×10^{-3} observable recombination events per cell per generation (Criss *et al.*, 2005) and may be higher (Rohrer *et al.*, 2005). Assuming that each pilin Av initiation event results in a structure that must be acted on by one of the HJ processing systems to maintain viability, we can use the RecA-dependent change in colony-forming unit (cfu) of the HJ-deficient strain to estimate the frequency of pilin Av initiation. The parental, HJ processing proficient strain produced about 1.05×10^6 cfu per colony after 24 h growth, which represents 20 generations, assuming each colony arose from a single cfu. *recG ruvB* and *recG ruvA* strains produced between 2% and 5% of the cfu per colony relative to the parental strain (Fig. 2B). To produce this observed reduction in cfu per colony after 20 generations one cfu would have to be lost every two or three generations. This calculation strongly suggests that pilin variation is initiated every second or third generation, but that only 1% of the time does initiation results in a variant *pilE*. We assume that this frequent initiation of pilin Av is necessary to accumulate the requisite level of diversity to continually propagate Gc within human populations. Any energy cost from frequent recombination initiation would be balanced by the selection for survival from immune surveillance.

The increasing appearance of antibiotic resistant gonococci and the loss of effective treatment strategies

have created a critical need for new ways to target antimicrobial agents. The requirement of HJ processing for Av, coupled with the significant inhibition of growth by mutation of both HJ processing pathways suggests that blocking HJ processing *in vivo* would inhibit growth of the organism. Furthermore, by blocking pilin Av, bacteria would be more antigenically stable and acquired immune responses would be more effective. As peptides inhibiting multiple HJ processing enzymes have been identified (Kepple *et al.*, 2005), a small molecule that could interfere with HJ processing may allow a unique type of antimicrobial to be developed. If efficacious in people or animals, these HJ processing inhibitors may also be useful against diseases caused by other bacteria with high-frequency Av systems such as Meningococcal meningitis or Lyme disease (van der Woude and Baumler, 2004).

Experimental procedures

Bacterial growth and genetics

E. coli and Gc strains were grown as previously described (Sechman *et al.*, 2005). Gonococcal strains were grown on Gc Medium Base (Difco) plus Kellogg supplements (GCB) [22.2 mM glucose, 0.68 mM glutamine, 0.45 mM cocarboxylase, 1.23 mM Fe(NO₃)₃; all from Sigma] (Kellogg *et al.*, 1963) at 37°C in 5% CO₂ or in GCB liquid (GCBL) medium [1.5% protease peptone no. 3 (Difco), 0.4% K₂HPO₄, 0.1% KH₂PO₄, 0.1% NaCl] with Kellogg supplements and 0.042% sodium bicarbonate. When appropriate 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Diagnostic Chemicals) was added to the media to induce expression from the *recA6* locus or the *nics6* complementing locus.

Molecular biology techniques were performed as previously described (Sechman *et al.*, 2005). *recG* and *ruvA* mutants were isolated as previously described (Sechman *et al.*, 2005). The Gc *ruvB* and *ruvC* loci were cloned into the pBLUNT vector (Invitrogen) using the primers RUVBFOR (CCATTCCGCCCGACATA), RUVBREV (GCTGATGTGGTCAACCCC), RUVCFOR2 (GGCGAATGTCGAAAACAATAAT), and RUVCREV2 (CAAATAATGCTTATTGCGGTAG) and mutated using a deletion/insertion strategy. Briefly, 925 bp of the *ruvB* gene was deleted using the BbsI (NEB) restriction endonuclease and an *ermC* cassette was inserted between the BbsI sites. The *ruvC* gene was mutated in a similar fashion; however, the 270 bp ClaI and NdeI (NEB) fragment was deleted and replaced with the *ermC* gene. Complementation was performed by amplifying fragments carrying the *ruvB* and *ruvC* genes from the Gc chromosome using primers RUVBFOR2 (TGCCGTCTGAAACGCGCCG), RUVBREV2 (CAAACGTCTGATAACAATGCCG), RUVCFOR3 (CTGGGACTGAACCGCAATAC) and RUVCREV3 (ATTTCATCTCGGTACACATTTTC) and expressing the wild-type genes from *lacI*O-regulated complementation locus. Double mutants were generated by transforming isolated genomic DNA from *ruvB* and *ruvC* mutants into *recG* and *ruvA* mutants. Southern blot and *pilE* sequence analysis was performed on all mutants.

Pilus-dependent colony morphology changes

Pilin Av was measured using the surrogate pilus-dependent colony morphology change assay (PDCMC). Gc were grown on agar medium and colony variation was scored after 22, 24, 26, 28 and 30 h of growth by observing the number of P⁻ outgrowths on the same three to seven colonies per time point using a stereomicroscope (Sechman *et al.*, 2005). No P⁻ outgrowths produced a score of 0, one P⁻ outgrowth a score of 1, etc. Colonies with four or more P⁻ sectors were given a score of 4 and the scores from individual colonies for each time point were averaged.

UV sensitivity

Gc were collected using a Dacron swab to a concentration of ~10⁸ cfu ml⁻¹, immediately serially diluted from 10⁻¹ to 10⁻⁶, plated on agar medium, and exposed to 0–80 mJ m⁻² UV radiation in a Stratlinker 1800. After 20 h of growth, colonies were counted and per cent survival was calculated.

DNA transformation

Transformation efficiency was assayed as previously described using pSY6 (Sechman *et al.*, 2005), with the modification of 50 µg ml⁻¹ of DNase I added to the cells 15 min after addition of transforming DNA and incubated for 10 min to degrade extracellular DNA. The transformation efficiency is the mean number of Nal^r transformants/cfu.

Bacterial growth in colonies

Gc colonies were grown on agar medium for 24 h. Five representative colonies were collected using sterile filter paper (Whatman) and placed into 1 ml of GCBL. After mixing vigorously, serial dilutions were spotted onto agar plates to determine cfu per colony.

Analysis of P⁻ colony variants for pilE deletion

Individual P⁻ variants that arose from different P⁺ progenitors were lysed as described (Sechman *et al.*, 2005) and PCR reactions performed with the *pilE* specific PILRBS and SP3A oligonucleotides (Wright *et al.*, 1994). The presence of a ~650 bp PCR product indicated that the *pilE* gene was intact, while the absence of that PCR product represented a *pilE* deletion.

Acknowledgements

We thank the members of the Seifert laboratory for comments on this work and manuscript. This work was supported by Public Health Service Grants R37 AI033493 and R01 AI044239 to H.S.S.

References

Asai, T., and Kogoma, T. (1994) Roles of *ruvA*, *ruvC* and *recG* gene functions in normal and DNA damage-inducible

- replication of the *Escherichia coli* chromosome. *Genetics* **137**: 895–902.
- Beam, C.E., Saveson, C.J., and Lovett, S.T. (2002) Role for *radA/sms* in recombination intermediate processing in *Escherichia coli*. *J Bacteriol* **184**: 6836–6844.
- Criss, A.K., Kline, K.A., and Seifert, H.S. (2005) The frequency and rate of pilin antigenic variation in *Neisseria gonorrhoeae*. *Mol Microbiol* **58**: 510–519.
- Howell-Adams, B., and Seifert, H.S. (2000) Molecular models accounting for the gene conversion reactions mediating gonococcal pilin antigenic variation. *Mol Microbiol* **37**: 1146–1159.
- Kellogg, D.S., Jr, Peacock, W.L., Deacon, W.E., Brown, L., and Pirkle, C.I. (1963) *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J Bacteriol* **85**: 1274–1279.
- Kepple, K.V., Boldt, J.L., and Segall, A.M. (2005) Holliday junction-binding peptides inhibit distinct junction-processing enzymes. *Proc Natl Acad Sci USA* **102**: 6867–6872.
- Kline, K.A., and Seifert, H.S. (2005a) Role of the Rep helicase gene in homologous recombination in *Neisseria gonorrhoeae*. *J Bacteriol* **187**: 2903–2907.
- Kline, K.A., and Seifert, H.S. (2005b) Mutation of the *priA* gene of *Neisseria gonorrhoeae* affects DNA transformation and DNA repair. *J Bacteriol* **187**: 5347–5355.
- Kline, K.A., Sechman, E.V., Skaar, E.P., and Seifert, H.S. (2003) Recombination, repair and replication in the pathogenic *Neisseriae*: the 3 R's of molecular genetics of two human-specific bacterial pathogens. *Mol Microbiol* **50**: 3–13.
- Lilley, D.M., and White, M.F. (2001) The junction-resolving enzymes. *Nat Rev Mol Cell Biol* **2**: 433–443.
- Meddows, T.R., Savory, A.P., and Lloyd, R.G. (2004) RecG helicase promotes DNA double-strand break repair. *Mol Microbiol* **52**: 119–132.
- Mehr, I.J., and Seifert, H.S. (1998) Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation, and DNA repair. *Mol Microbiol* **30**: 697–710.
- Michel, B. (2000) Replication fork arrest and DNA recombination. *Trends Biochem Sci* **25**: 173–178.
- Morimatsu, K., and Kowalczykowski, S.C. (2003) RecFOR proteins load RecA protein onto gapped DNA to accelerate DNA strand exchange. A universal step of recombinational repair. *Mol Cell* **11**: 1337–1347.
- Morton (1977) Gonorrhoea in earlier times. In *Gonorrhoea*, Vol. 9. Morton, R.S. (ed.). London: W.B. Saunders, pp. 1–24.
- Rohrer, M.S., Lazio, M.P., and Seifert, H.S. (2005) A real-time semi-quantitative RT-PCR assay demonstrates that the *pilE* sequence dictates the frequency and characteristics of pilin antigenic variation in *Neisseria gonorrhoeae*. *Nucleic Acids Res* **33**: 3363–3371.
- Rothenberg, R.B. (1993) Diseases of western antiquity. In *Cambridge World History of Human Disease*. Kiple, K.F. (ed.). Cambridge: Cambridge University Press, pp. 756–763.
- Sanchez, H., Kidane, D., Reed, P., Curtis, F.A., Cozar, M.C., Graumann, P.L., *et al.* (2005) The RuvAB branch migration translocase and RecU Holliday Junction resolvase are

- required for double-stranded DNA break repair in *Bacillus subtilis*. *Genetics* **171**: 873–883.
- Sechman, E.V., Rohrer, M.S., and Seifert, H.S. (2005) A genetic screen identifies genes and sites involved in pilin antigenic variation in *Neisseria gonorrhoeae*. *Mol Microbiol* **57**: 468–483.
- Seifert, H.S. (1997) Insertionally inactivated and inducible *recA* alleles for use in *Neisseria*. *Gene* **188**: 215–220.
- Seifert, H.S., Wright, C.J., Jerse, A.E., Cohen, M.S., and Cannon, J.G. (1994) Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *J Clin Invest* **93**: 2744–2749.
- Sharples, G.J., Ingleston, S.M., and Lloyd, R.G. (1999) Holliday junction processing in bacteria: insights from the evolutionary conservation of RuvABC, RecG, and RusA. *J Bacteriol* **181**: 5543–5550.
- Skaar, E.P., Lazio, M.P., and Seifert, H.S. (2002) Roles of the *recJ* and *recN* genes in homologous recombination and DNA repair pathways of *Neisseria gonorrhoeae*. *J Bacteriol* **184**: 919–927.
- Stein, D.C., Danaher, R.J., and Cook, T.M. (1991) Characterization of a *gyrB* mutation responsible for low-level nalidixic acid resistance in *Neisseria gonorrhoeae*. *Antimicrob Agents Chemother* **35**: 622–626.
- Stohl, E.A., and Seifert, H.S. (2001) The *recX* gene potentiates homologous recombination in *Neisseria gonorrhoeae*. *Mol Microbiol* **40**: 1301–1310.
- Sun, Y.H., Exley, R., Li, Y., Goulding, D., and Tang, C. (2005) Identification and characterization of genes required for competence in *Neisseria meningitidis*. *J Bacteriol* **187**: 3273–3276.
- Tobiason, D.M., and Seifert, H.S. (2006) The diplococcus, *Neisseria gonorrhoeae*, is polyploid. *PLoS Biol* **4**: (in press).
- Wain, B.S. (1947) *The Unconquered Plague*. New York: International University Press.
- van der Woude, M.W., and Baumber, A.J. (2004) Phase and antigenic variation in bacteria. *Clin Microbiol Rev* **17**: 581–611.
- Wright, C.J., Jerse, A.E., Cohen, M.S., Cannon, J.G., and Seifert, H.S. (1994) Nonrepresentative PCR amplification of variable gene sequences in clinical specimens containing dilute, complex mixtures of microorganisms. *J Clin Microbiol* **32**: 464–468.