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Origin, evolution, and success of pbla, the gonococcal beta-lactamase plasmid, and implications for public health

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Abstract:	Neisseria gonorrhoeae is a leading cause of sexually transmitted infection (STI) and a priority AMR pathogen. Two narrow host range plasmids, pbla and pConj, have contributed to ending penicillin and tetracycline therapy, respectively, and undermine current prevention strategies including doxycycline post-exposure prophylaxis (Doxy-PEP). Here, we investigated the origin and evolution of the beta-lactamase plasmid, pbla. We demonstrate that pbla was likely acquired by the gonococcus from Haemophilus ducreyi, and describe the subsequent evolutionary pathways taken by the three major pbla variants. We show that the ability of pConj to spread pbla promotes their co-occurrence in the gonococcal population and the spread of pbla. Changes that mitigate fitness costs of pbla and the emergence of TEM beta-lactamases that confer increased resistance have contributed to the success of pbla. In particular, TEM-135, which has arisen in certain pbla variants, increases resistance to beta-lactams and only requires one amino acid change to become an extended spectrum beta-lactamase (ESBL). The evolution of pbla underscores the threat of plasmid-mediated resistance to current therapeutic and preventive strategies against gonococcal infection. Given the close relationship between pbla and pConj, widespread use of Doxy-PEP is likely to promote spread of both plasmids, strains which carry pConj and are resistant to third generation cephalosporins, and the emergence of plasmid-mediated ESBL in the gonococcus, with significant public health consequences.
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Responses to the reviewers' general comments

We are grateful for the supportive comments made by all the reviewers about our manuscript, and its relevance to AMR in the gonococcus and public health.

However, each reviewer expressed concern about whether we can be certain about the number of times *N. gonorrhoeae* acquired p*bla* from *Haemophilus* spp.. Two acquisitions is the most parsimonious explanation for our data, but we agree with the reviews that it is not the only one.

If we redrew the tree with both plasmids (as suggested by reviewers 1 and 3), we would still observe two roots, but this does not necessarily prove there were two separate plasmids introductions. Given the difficulty in imputing evolutionary origins due to i) databases mostly including recent isolates, ii) the close relationship of p*bla* in *Haemophilus* and the gonococcus, and iii) the paucity of available whole genome sequences for *H. ducreyi* (only 31 as of 8th April 2025), we have decided to be cautious about estimating the number of introductions of the plasmid into *N. gonorrhoeae*.

We now also include detailed inspection of Tn*2* truncations in gonococcal plasmids (compared with *H. ducreyi* p*bla,* new Supplementary Figure 1A line 130 - 133), and alleles around the deletion that differentiates p*bla*.1 from p*bla*.2 in *N. gonorrhoeae* and *H. ducreyi* (new Supplementary Figure 1B, line 134 - 138). However, these analyses are not conclusive about the number of introductions of p*bla* into the gonococcus. Therefore, we have altered the manuscript at several points in line with the reviewers' concerns (*e.g.* lines 25, 50, 138 and 310 - 315).

Reviewer #1:

My only major concerns with the manuscript are related to the evolutionary analyses and prevalence estimates based on genomic data.

1. The authors state that pbla.1 and pbla.2 were separately introduced to the N. gonorrhoeae population from H. ducreyi. This argument would be strengthened if a metric for relatedness was included on Line 109, and if both H. ducreyi plasmids (or even better a diverse collection of H. ducreyi plasmids) were included in the phylogeny in Figure 1B. I would expect that two separate introductions would result in the pbla.1 and pbla.2/pbla.3 clades separately nesting within H. ducreyi plasmid diversity.

Please see our comments above.

2. The authors present an analysis of the prevalence of pbla types across time based on whole genome sequencing data in pubMLST. However, there are very few representative, longitudinal studies of N. gonorrhoeae diversity. Most available datasets only span a few years (e.g. GISP, Euro-GASP, genomic epidemiology studies from Victoria, Australia). Even within a gonococcal lineage (e.g. Figure 4E), the isolates collected from an earlier time point could be from a completely different study, sampling scheme, or geographic region than those collected in 2018-2019. Without a representative, longitudinal sample, a phylodynamic analysis is required to demonstrate that particularly lineages are expanding or becoming extinct.

The reviewer raises the valid point that available datasets for the gonococcus are neither longitudinal or systematically collected at a national level, unlike some collections of whole sequences of *N. meningitidis* https://www.meningitis.org/our-work/action-and-support/mrf-meningococcus-genome-library. We therefore highlight the correlative nature of this association. However, we have left these data in our revised manuscript (adding comments to reflect this issue, line 348/349 in discussion) as they are consistent the low frequency of p*bla*.2 in all available genome sequences in PubMLST (>15,000 isolates) and a recent longitudinal study of > 1,700 gonococcal isolates from a single city in China, Guangzhou, which described a marked increase in p*bla*.1 (p*bla* Africa, 18.42% to over 90% of isolates) and a concurrent fall in p*bla*.2 infections (from 81.6 to 7.6%) over a 10 year period. We now discuss this paper (which was published after our initial submission) in our revised manuscript (line 349 -352).

Minor points

Lines 102-103: The NEIS nomenclature may not be familiar to those outside the Neisseria field. It would be helpful to have a short description of what the genes are predicted to encode here.

We now describe their predicted function (line 105, *repA* and *mobA*).

Lines 107/365: Are the sequences publicly available?

Yes they are and we now provide identifiers from PubMLST so than can be accessed simply by readers and reviewers (line 110).

Line 132: Extra word in "mobilised by at a frequency"?

Thank you. We have changed the text (line 155).

Line 168: What is the NEIS locus number for mobA?

NEIS2961; this is now given at relevant places in our manuscript (line 105, 201).

Line 177/325: Similar to the NEIS nomenclature, core genome MLST may not be familiar to readers outside the N. gonorrhoeae filed, and many N. gonorrhoeae studies do not use this method for clustering. An explanation of what this nomenclature indicates would be useful to the reader.

Thank you. Details of this scheme have been included in our revised manuscript (line 214 - 216).

Line 203: It would be helpful to highlight these strains in supplementary figure 2 to further demonstrate they are in distinct N. gonorrhoeae lineages.

(line 255).

Figures 2/3: Given the small number of observations, I think having only the dot plot rather than the dot plot and a box plot makes sense. Also, are there relevant limits of detection that could be added to Figure 2?

Thank you. As suggested the limit of detection has been added to these figures. We decided to keep the boxplot representation as we feel it aids visualisation.

Reviewer #2:

We are grateful for the reviewer's supportive general comments.

It is unclear from the text and Fig. 1 how the authors differentiated between two independent acquisitions of H. ducreyi plasmids and pbla.1 plasmids arising from deletions in pbla.2. Fig. 1A makes it appear that a deletion between the two copies of NEIS2964 in pbla.2 would generate pbla.1. The text states that different truncations of Tn2 occurred in the plasmids (line 123), but that is not apparent from Fig. 1A. More information in the text or a change in the figure would be helpful.

Please see our comments above. We have analysed the truncation/deletion and surrounding sequences in detail. Results are inconclusive.

Minor points

1. The color of the star and square symbols in Fig. 1B is very similar, and since the symbols are so small, it is difficult to differentiate them. Change the color or fill of the star or square.

We have changed the colours as suggested; this has improved the clarity of the figure.

2. Indicate in the Fig. 3 legend how many repeats were performed of the western blot in Fig. 3D so that the reader will not have to dig through the supplementary figures to find out that the experiment was repeated.

Experiments were performed on three independent occasions as now described in the legend of Figure 3.

3. It is unclear if the sentence in line 263 is meant to indicate that pbla might already be in some NmUC strains and the authors just didn't detect it, that pbla will appear in NmUC in the future, or that NmUC is capable of supporting pbla. The ambiguity is OK, but if the authors meant one of those things in particular, they should state it clearly.

We have clarified this point; p*bla* has not been found in the whole genome sequences of NmUC strains (line 322/325 and (1)).

4. Reword line 292.

We have re-written this section (line 362/363).

Reviewer #3:

We are grateful for the reviewer's supportive comments.

1) A major concern is the breadth of comparison in analyzing pbla's origin and evolution. While Haemophilus spp. are known to harbor plasmids related to pbla, previous studies have primarily focused on H. ducreyi DMC64. This narrow focus does not fully address the broader evolutionary history of pbla beyond Haemophilus strains or consider potential alternative sources. Expanding the comparative analysis could strengthen the evolutionary conclusions regarding whether pbla was acquired by the gonococcus from Haemophilus specifically.

Please see our comments above.

2) A major concern is the mechanistic uncertainty in pbla transfer. While the study like previous publication demonstrates that pbla and pConj frequently co-occur, it does not explicitly establish how pConj facilitates pbla transfer. It remains unclear whether pbla is directly mobilized by pConj or whether their co-residence in the same bacterial cell passively increases the likelihood of co-inheritance. Additionally, previous studies have already established that pbla and pConj together contribute to increased infection, and the specific novelty this study adds beyond prior work could be more clearly articulated.

Our work shows for the first time that p*bla* is a mobilizable plasmid that relies on its own relaxase and not the relaxase encoded by pConj. For clarity, we have now outlined how conjugative plasmids can mediate the transfer of mobilisable plasmids before we describe the construction and mobilisation of isogenic plasmids between isogenic strains (line 193 - 198). Previous work has been performed with naturally occurring plasmid variants, often using donor/recipients of different genetic backgrounds ((2-4)). Therefore, we think our work adds mechanistic insights into p*bla* transfer and highlight this in our revised manuscript (line 203 - 208).

Minor points

1) pbla variants may exhibit different fitness costs or mobilization mechanisms depending on their genetic background, which could impact the generalizability of the findings. The authors effectively establish that the higher copy number associated with pbla.2 could impose a fitness cost. They propose that successful pbla variants have evolved to mitigate these costs, explaining why pbla.1 and pbla.3 (once they acquired penicillin resistance) outcompeted pbla.2. However, this conclusion scaling this to isolate population shifts is largely based on correlation, as there is no accompanying data on penicillin use driving this selection. While this may be beyond the scope of the current study, adding a discussion point to bridge this logical connection would help readers better contextualize the findings.

We are grateful for the reviewer's interesting comment and included discussion of this issue to our revised manuscript (line 363/364).

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Origin, evolution, and success of p*bla*, the gonococcal beta-lactamase plasmid, and implications for public health

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ABSTRACT

Neisseria gonorrhoeae **is a leading cause of sexually transmitted infection (STI) and a priority AMR pathogen. Two narrow host range plasmids, p*bla* and pConj, have contributed to ending penicillin and tetracycline therapy, respectively, and undermine current prevention strategies including doxycycline post-exposure prophylaxis (Doxy-PEP). Here, we investigated the origin and evolution of the beta-lactamase plasmid, p*bla*. We demonstrate that p*bla* was likely acquired by the gonococcus from *Haemophilus ducreyi*, and describe the subsequent evolutionary pathways taken by the three major p*bla* variants. We show that the ability of pConj to spread p*bla* promotes their co-occurrence in the gonococcal population and the spread of p*bla*. Changes that mitigate fitness costs of p*bla* and the emergence of TEM beta-lactamases that confer increased resistance have contributed to the success of p*bla*. In particular, TEM-135, which has arisen in certain p*bla* variants, increases resistance to beta-lactams and only requires one amino acid change to become an extended spectrum beta-lactamase (ESBL). The evolution of p*bla* underscores the threat of plasmid-mediated resistance to current therapeutic and preventive strategies against gonococcal infection. Given the close relationship between p*bla* and pConj, widespread use of Doxy-PEP is likely to promote spread of both plasmids, strains which carry pConj and are resistant to third generation cephalosporins, and the emergence of plasmid-mediated ESBL in the gonococcus, with significant public health consequences.**

AUTHOR SUMMARY

Neisseria gonorrhoeae is a threat to sexual and reproductive health. With no available vaccine against gonococcal disease, control of infection depends on having effective antimicrobials for the treatment of cases and their contacts. However, strains of *N. gonorrhoeae* have acquired resistance to all classes of antibiotics, including third generation cephalosporins, the mainstay of treatment for gonococcal disease. The gonococcus can carry two resistance plasmids, the conjugative plasmid pConj and beta-lactamase plasmid p*bla*, which have rendered treatment with tetracycline/doxycycline and penicillin ineffective, respectively. Here, we investigated the origin and evolution of p*bla,* and the molecular basis of its success in the gonococcal population. We show that p*bla* was likely acquired from *Haemophilus ducreyi* and has adapted to the gonococcus both through gene loss that reduces fitness costs, and through mutations that increase resistance and favour the development of an extended spectrum beta-lactamase. Our findings show that the ability of pConj to mobilise p*bla* promotes their co-occurrence, highlighting the potential to co-select for p*bla* by favouring pConj-containing strains through doxycycline use. Our results highlight the continuing adaptation of p*bla* to its host, which could undermine treatment options in the future.

INTRODUCTION

Neisseria gonorrhoeae causes ~80 million cases of sexually transmitted infection (STIs) annually (4) and is a WHO priority pathogen due to its extensive antimicrobial resistance (AMR) (5). *N. gonorrhoeae* has two resistance plasmids, pConj and p*bla*, which contributed to the cessation of tetracycline and penicillin therapy, respectively. Furthermore, pConj carrying *tetM* confers resistance to doxycycline as well as tetracycline, so undermines the ability of doxycycline post-exposure prophylaxis (Doxy-PEP) to prevent gonococcal infection (6, 7). pConj and p*bla* are highly prevalent in low and middle-income countries (LMICs) where syndromic treatment of STIs with doxycycline has been recommended (1, 7, 8). Therefore, it is important to understand the factors driving the success of these plasmids in gonococcal populations.

pConj is a 39-42 kb conjugative plasmid, that can confer high-level tetracycline/doxycycline resistance (7, 9), and can be categorised into seven variants (8). p*bla* emerged in the gonococcus in the 1970s and encodes a TEM beta-lactamase conferring penicillin resistance (10, 11). p*bla* TEM beta-lactamases require a few amino acid changes to become an extended-spectrum beta-lactamase (ESBL) (12), which would render third-generation cephalosporins, the current first-line treatment, ineffective (13). p*bla* is usually closely associated with pConj which can mobilise p*bla* (8, 14); around 85% of strains that harbour p*bla* also contain pConj.

There are three main p*bla* variants, characterised by the presence/absence of certain genes (1). The 7.4 kb p*bla*.2 (also referred to as p*bla* Asia) has been considered the ancestral

plasmid (15). p*bla*.1 (5.6 kb, p*bla* Africa) is the commonest variant and has a deletion in the replication region, while p*bla*.3 (5.1 kb, p*bla* Rio/Toronto) lacks the region implicated in p*bla* mobilisation (1, 15). Variants of p*bla* are associated with certain pConj variants and TEM alleles (1). p*bla*.1 mostly carries TEM-1 or TEM-1_{P14S}, while p*bla*.3 is associated with TEM-135; p*bla*.2 carries TEM-1 or TEM-135. Importantly, the M182T substitution in TEM-135 is a 'stepping stone' mutation before the enzyme becomes an ESBL (12, 16).

Here, we investigated the origin, evolution and characteristics of the three p*bla* variants. We demonstrate that the spread and distribution of p*bla* in gonococci results from the dynamics of its association with pConj, fitness costs, and resistance levels. p*bla* is found with mobile pConj variants and has evolved to avoid fitness costs and confer higher resistance to beta-lactams. Our results underline the threats posed by p*bla* and pConj, particularly if their spread and prevalence in the gonococcal population is promoted by the widespread implementation of Doxy-PEP.

RESULTS

p*bla* has been acquired by the gonococcus from *Haemophilus*

*Haemophilu*s spp. are known to harbour plasmids related to p*bla* (17-22); however, without nucleotide sequence data, these early studies could not characterise the precise relationship between *Haemophilus* and *Neisseria* beta-lactamase plasmids. Therefore, we interrogated *Haemophilus* whole genome sequences (WGS) in the PubMLST database (23) (6,896 isolates, 12 species, Supplementary Table 1) for p*bla*. We searched for Tn*2* as p*bla* TEM-1b is located on this transposon (24, 25), and confirmed the presence of p*bla* replicons by searching for NEIS2960, NEIS2358 (*repA*), and NEIS2961 (*mobA*) (1). Tn*2* is present in 12.5% of *Haemophilus influenzae* (6,403 isolates) and 19.3 % *Haemphilus parainfluenzae* (269 isolates), while p*bla*-like replicons were detected in 0.3 % of *H. influenzae* and 1.5% *H. parainfluenzae* isolates. However, Tn*2* and p*bla* sequences only co-occurred in two isolates of *H. influenzae* (PubMLST ids: 23482 and 33361) and *H. parainfluenzae* (PubMLST ids: 16289 and 34872).

In contrast, seven of 31 (22.6%) *H. ducreyi* isolates (Supplementary Table 2) harbour TEM-1 containing p*bla*-like plasmids. *H. ducreyi* strains can be divided into two clades/classes (26), and a p*bla.*1like plasmid in Class I isolate and a p*bla.*2-like plasmid in a Class II isolates. The sequences of plasmids from *H. ducreyi* HD183 (Class I, 9.1 kb) and DMC64 (Class II, 10.9 kb) (27) were aligned to gonococcal p*bla*.1 and p*bla*.2, and were found to be highly similar (> 83% , Figure 1A). p*bla*.1 and the 9.1 kb *H. ducreyi* plasmid carry *repB* and one copy of NEIS2964. Both the 10.9 kb *H. ducreyi* plasmid and gonococcal p*bla*.2 carry two *rep* alleles, *repA* and *repB,* as well as two copies of NEIS2964. The major difference is that Tn*2* is intact in

the *H. ducreyi* plasmids while gonococcal p*bla* lack *tnpA* and have a truncated *tnpR.* These findings are consistent with p*bla* transfer from *H. ducreyi* into the gonococcus with the event associated with truncation of Tn*2*.

Two alternative scenarios can explain the occurrence of two distinct p*bla* variants in both *H. ducreyi* and *N. gonorrhoeae*: (1) independent introductions of p*bla*.1 and p*bla*.2, with both introductions associated with a truncation of Tn*2*, or (2) introduction of p*bla*.2 into the gonococcus with p*bla*.1 emerging independently in *H. ducreyi* and *N. gonorrhoeae* through the deletion of *repB* and one copy of NEIS2964. Comparison of the Tn*2* deletion site in gonococcal p*bla* variants demonstrates that the plasmids carry distinct *tnpR* alleles (allele 3 and 2 for p*bla*.1 and p*bla*.2, respectively), as their truncations differ by a single nucleotide (Supplementary Figure 1A), supporting the hypothesis of two independent Tn*2* truncations/introductions. We next examined the p*bla*.1 deletion site (spanning *repB* and one copy of NEIS2964). *H. ducreyi* p*bla*.2 carries two copies of NEIS2964 (alleles 2 and 3) which differ by three nucleotides (Supplementary Figure 1B). Gonococcal p*bla*.2 carries alleles 1 and 2. *H. ducreyi* p*bla*.1 carries allele 3, whereas gonococcal p*bla*.1 is associated with allele 2, suggesting p*bla*.1 arose independently in *H. ducreyi* and *N. gonorrhoeae*. Therefore, analysis of Tn*2* truncations and p*bla* deletions is inconclusive about whether a single or multiple introductions of p*bla* occurred into the gonococcus.

To investigate the evolutionary relationships between gonococcal p*bla* variants further, we examined a subset of p*bla* (414 of 2,758, Supplementary Table 3) (1) with the 10.9 kb *H. ducreyi* p*bla* as reference; plasmids were from 1979-2022 with the same proportion of variants as the whole population (*i.e.* 70% p*bla*.1, 14% p*bla*.2, 16% p*bla*.3) (1). A maximum

likelihood phylogeny placed p*bla* variants into distinct clades, with p*bla*.1 split from the other variants. While *H. ducreyi* p*bla* carry TEM-1, TEM-1_{P14S} arose in p*bla*.1 while TEM-135 arose in p*bla*.2, with p*bla*.3 emerging from TEM-135 carrying p*bla*.2 (Figure 1B).

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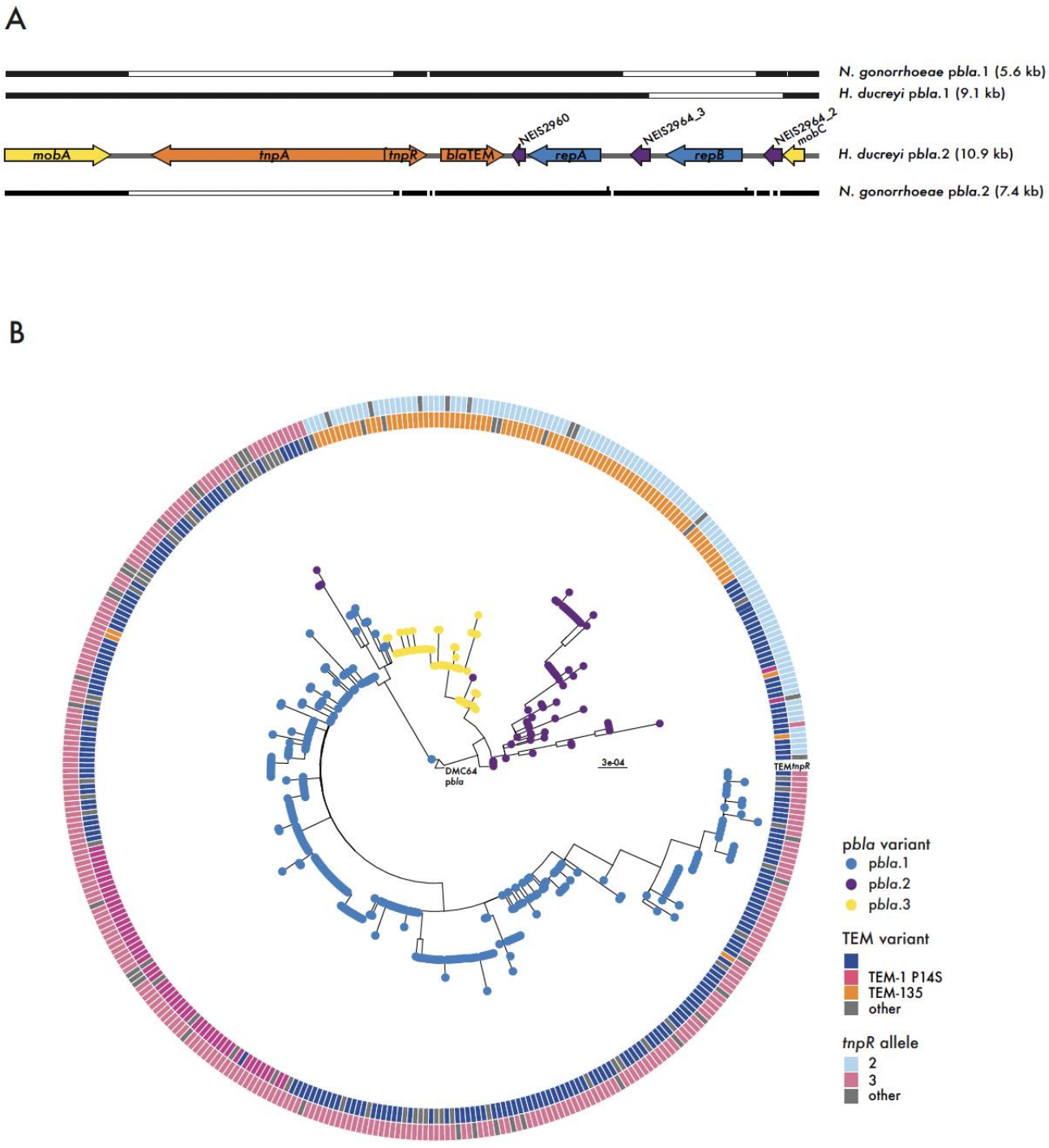

Figure 1: Evolutionary relationships between p*bla* variants. (A) Schematic representation of alignment of gonococcal and *H. ducreyi* p*bla* variants to *H. ducreyi* p*bla*.2. Aligned regions are represented as black bars, with deletion regions and nucleotide polymorphisms indicated in white within. Insertions are shown as black lines above the bars. ORFs on *H. ducreyi* p*bla*.2 are coloured according to gene function; yellow, mobilisation proteins; orange, Tn*2*-derived genes including *bla*TEM; light blue, replication initiation proteins; purple, other gene function. (B) Maximum likelihood tree of 414 gonococcal p*bla* sequences with tips coloured according to p*bla* variant; the tree was rooted at *H. ducreyi* p*bla*.2. Circles indicate the *tnpR* allele and the TEM variant carried.

p*bla* is associated with pConj variants that promote its spread

To understand the association between p*bla* and pConj, we examined the ability of different pConj variants to transfer p*bla*. Initially, matings were performed between isogenic strains (FA1090 or 2086_K) to eliminate any barrier to horizontal gene transfer between unrelated strains (28). Additionally, Δ*pilD* donors and recipients were constructed to block transformation (29-31), and the transfer p*bla*.1 by pConj.1, the commonest combination of these plasmids (1), was measured. p*bla*.1 was mobilised at a frequency of ~1% transconjugants/recipient for FA1090 and 2086_K (Supplementary Figure 1), while no p*bla* transfer was detected in the absence of pConj.

Conjugative plasmids can block the acquisition of other plasmids by expressing entry exclusion proteins (32). Entry exclusion could impede p*bla* mobilisation, as the initial transfer of pConj during conjugation could block subsequent acquisition of p*bla*. pConj encodes a predicted lipoprotein annotated as TrbK entry exclusion protein (33), with 21% amino acid similarity with *Agrobacterium fabrum* Ti plasmid TrbK (locus tag: ATU_RS23180, Genbank: NC_003065.3). To examine whether pConj in a recipient can impair the transfer of p*bla*, we compared the mobilisation rates of p*bla* into pConj-free and pConj-harbouring recipients. There was no difference in p*bla* transfer into pConj-free (1.1%) and pConj-containing recipients (0.9%, Welch two-sample t-test, p = 0.87, Supplementary Figure 1), indicating that surface exclusion is unlikely to limit p*bla* spread.

We next evaluated p*bla* mobilisation by pConj variants that are frequently associated with p*bla* (*i.e.* pConj 1, 3, and 4, Figure 2A) and by variants that are infrequently associated with p*bla* (*i.e.* pConj.2 and 7). The conjugation frequencies of pConj.1, 3 and 4 were >79% (Figure

2B) but several orders of magnitude lower for pConj.2 and pConj.7 which are not associated with p*bla* (Figure 2A). The rate of p*bla* mobilisation correlated with conjugation frequencies (Figure 2B), with p*bla* transfer by pConj.2 undetectable in our assays (limit of detection, L.O.D. = 0.001%). Taken together, results indicate that p*bla* is associated with pConj variants that mobilise it efficiently and promote its spread in the gonococcal population.

The immobility of p*bla*.3 is reflected in its restricted distribution

There are conflicting data about the mobility of p*bla*.3 (34-36). Therefore, we assessed the mobilisation of wild-type p*bla* variants by pConj.1. Results indicate that wild-type p*bla*.1 and p*bla*.2 were mobilised efficiently (transfer rate, ~ 1%), while p*bla*.3 mobilisation was not detected (Figure 2C). To assess whether p*bla* variant deletions are responsible for these differences, we introduced variant-specific deletions into p*bla*.2, generating the isogenic plasmids p*bla*.1^{iso} and p*bla*.3^{iso}. Mobilisation of the isogenic plasmids did not differ from wild-type plasmids (p = 0.82, Figure 2C), indicating the 2.3 kb deletion in p*bla*.3 compared with p*bla*.2 is responsible for the lack of p*bla*.3 transfer. The immobility of p*bla*.3 is evident from its restricted distribution in three related lineages, whilst p*bla*.1 and p*bla*.2 are found across the gonococcal population (Supplementary Figure 3) (1).

Mobilisable plasmids deploy diverse strategies to exploit the conjugative machinery of co-existing conjugative plasmids (37). Some mobilisable plasmids encode their own relaxase which recognises and nicks their origin of transfer (*oriT*) and then guides the plasmid DNA through the Type 4 secretion system encoded by the conjugative plasmid. Alternatively, the *oriT* of mobilizable plasmids can be recognised by the relaxase encoded by a conjugative plasmid. Therefore, we next examined the genes responsible for the immobility of p*bla*.3 by

196 generating isogenic p*bla*.2 mutants lacking genes absent in p*bla*.3, and assessing their ability
197 to be mobilised between *pilD* mutants of FA1090 by pConj1. The p*bla*.3-characteristic
198 deletion spans *mobA* (NEIS2961, encoding the relaxase (38)), *mobC* (NEIS2962) and
199 NEIS2964. Deletion of *mobA* abolished p*bla* transfer (Figure 2D), indicating that p*bla*
200 mobilisation depends on its own relaxase and not the pConj relaxase. Removal of NEIS2962
201 significantly reduced p*bla* mobilisation ($p = 0.01$, Figure 2D). NEIS2962 is related to MobC
202 from *E. coli* plasmid RSF1010, which unwinds DNA at the *oriT* (39). NEIS2962 homodimers
203 are structurally related to MobC and predicted to recognise the p*bla oriT* but not a
204 scrambled *oriT* sequence (Supplementary Figure 4). Deletion of both copies of NEIS2964 did
205 not impact p*bla*.2 transfer.

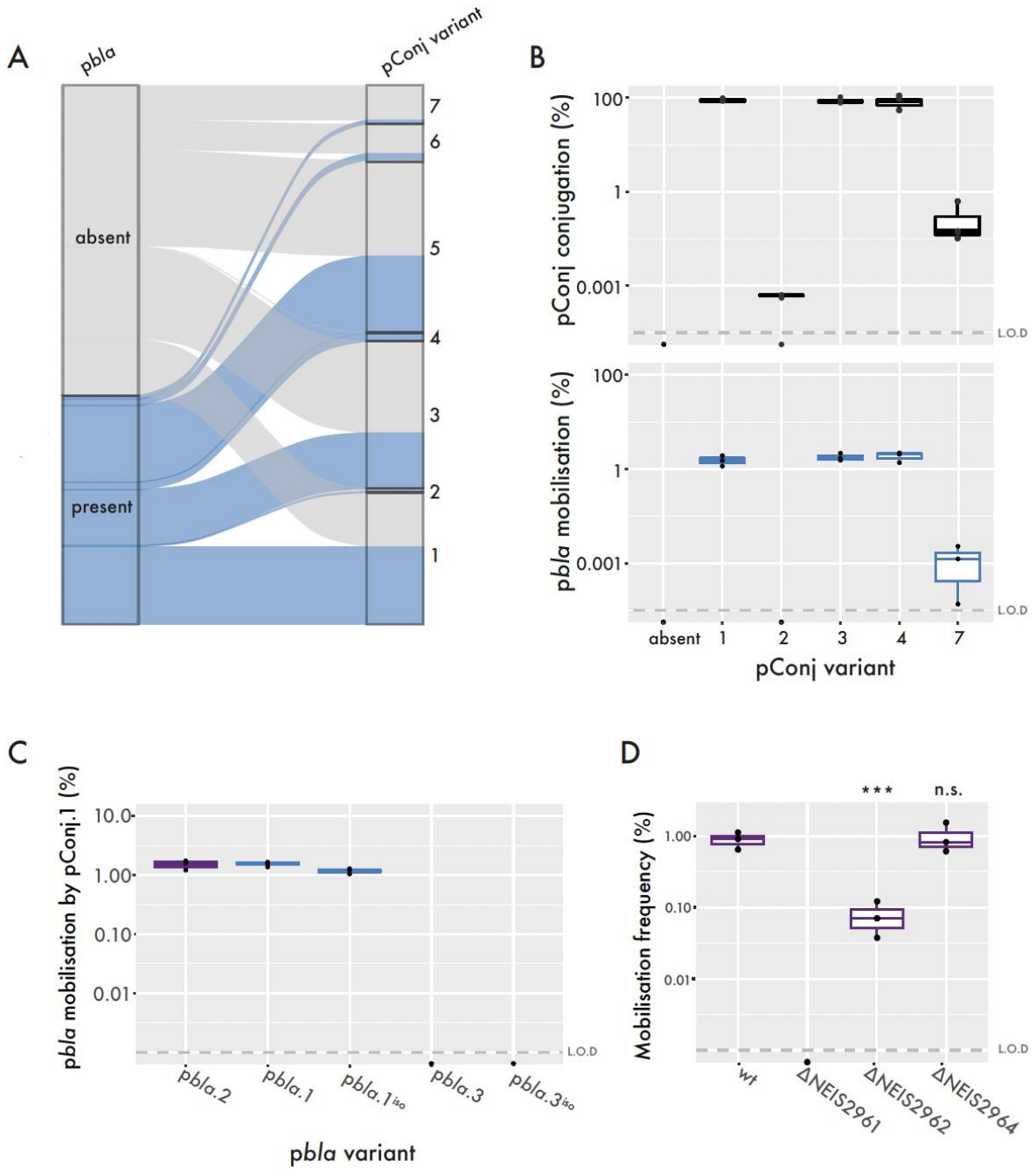

Figure 2: p*bla* mobilisation by pConj. (A) Sankey plot of pConj carrying isolates (n = 4,883 isolates) (1), displaying the presence of p*bla* (left) and co-occurrence of p*bla* with individual pConj variants (right). (B) Conjugation rates of pConj variants (top) and the mobilisation rates of co-located p*bla*.1 (bottom). The limit of detection (L.O.D.) is indicated as a dashed line. (C) Mobilisation rates of wild type and isogenic p*bla* variants (p*bla*^{iso}) by pConj.1. (D) The impact of single *mob* gene knockouts in p*bla*.2 on p*bla* mobilisation frequencies. All assays consist of three individual repeats and were analysed by one-way ANOVA with Tukey multiple comparisons; n.s. p>0.05, *** p<0.001.

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TEM-135 confers increased penicillin resistance

If a plasmid is beneficial to its host, one would expect that plasmid carrying isolates to be highly prevalent in a lineage, due to their competitive advantage of isolates without the plasmid. Therefore, to assess the prevalence of p*bla* in certain lineages, we resolved the gonococcal population structure by clustering isolates according to allelic differences in loci that are core to the gonococcus (40), and defined *N. gonorrhoeae* core genome clusters (Ng_cgc$_{400}$) with a cut-off of 400 allelic differences. Although p*bla*.3 is immobile, it is highly prevalent in Ng_cgc$_{400}$s 25 and 298 with 56.8 and 39.6% of isolates in these lineages carrying p*bla*, respectively, Table 1, Supplementary Figure 3) (1). This suggests p*bla*.3, which carries TEM-135, confers a benefit to the gonococcus that has led to the clonal expansion of isolates containing this p*bla* variant. To test this, we measured the penicillin MICs conferred by p*bla* variants. Whilst TEM-1 carrying p*bla*.1 and 2 conferred MICs of 8 µg/ml, p*bla*.3 with TEM-135 conferred a significantly higher MIC (32 µg/ml, p=0.003, Figure 3A). To establish whether the TEM variant determines resistance levels, we changed p*bla*.3 TEM-135 into TEM-1 by introducing a T182M substitution. This substitution reduced the MIC conferred by p*bla*.3 to levels of p*bla*.1 and p*bla*.2, demonstrating that TEM-135 confers elevated MICs (Figure 3B). We also compared resistance conferred by TEM-1, TEM-1$_{P14S}$ and TEM-135 (which together account for >95% of gonococcal TEMs (1)) expressed by p*bla*.2. Again, TEM-135 significantly increased MICs (128 µg/ml *vs.* 8 µg/ml with TEM-1, p<0.001, Figure 3C).

To explore the basis for the different MICs, we assessed cellular TEM levels. Levels of mature TEM-135 (29 kDa) were significantly higher than TEM-1 or TEM-1$_{P14S}$ (Figure 3D, Supplementary Figure 5), consistent with increased stability of TEM-135 (16). In conclusion, the appearance of TEM-135, particularly associated with p*bla*.3, provides a significant

benefit to the gonococcus by enhancing resistance against beta-lactams, with MICs correlating with cellular TEM levels.

Table 1: The percentage of isolates carrying p*bla* in three largest Ng_cgc400 that carry each variant.

Ng_cgc$_{400}$	**Number of isolates**	**p*bla* variant(s)**	**p*bla* carriage (%)**
21	574	1	64.3
33	476	1	51.9
187	94	1	62.8
3	4545	1 / 2 / 3	1.2 / 0.5 / 0.3
29	473	1 / 2	14 / 18
175	318	2	8.5
25	346	3	56.8
298	101	3	39.6
391	31	3	87.1

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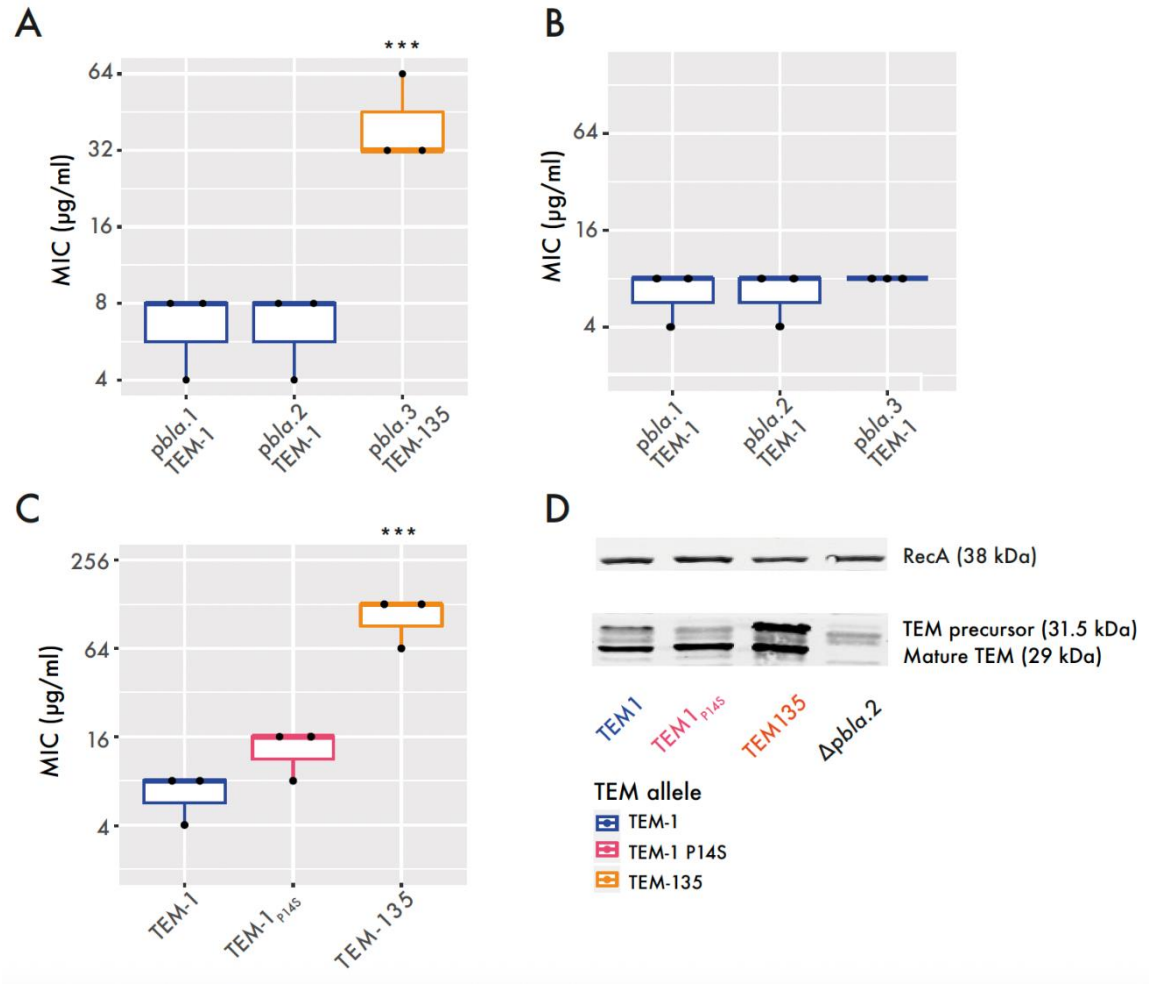

Figure 3: TEM-135 increases the MIC. (A) Penicillin G MICs of p*bla* variants in FA1090 isogenic strain background (one-way ANOVA on $\log_2$-transformed MIC values with Tukey multiple comparisons of means; *** p<0.001). (B) MICs of TEM-1 in different p*bla* variant backbones. (C) MICs of different TEM variants in p*bla*.2 backbone (one-way ANOVA on $\log_2$-transformed MIC values; *** p<0.001). (D) Cellular levels of TEM variants were assessed by Western blot analysis. The image is representative of three biological repeats.

Successful p*bla* variants have evolved with reduced fitness costs

Plasmids often impose fitness costs, disadvantaging isolates that carry plasmids (41). We therefore assessed the fitness costs of p*bla* by introducing p*bla*.1 into isolates from a range of lineages (Supplementary Figure 3) and competing plasmid-carrying *vs.* plasmid-free strains over 24 hrs. p*bla*.1 had no detectable fitness cost in any isolate (Figure 4A), consistent with its continued prevalence in the gonococcus (Figure 4B). We also compared the fitness costs of the three p*bla* variants in wild-type FA1090. In contrast to p*bla*.1 and p*bla*.3 which impose no fitness cost, p*bla*.2 inflicts a significant fitness cost which was evident within 24 hrs (Figure 4C). This is associated with a higher copy number for p*bla*.2 (>6 copies/chromosome) than the other variants (1-2 copies/chromosome, Figure 4D).

The fitness costs of p*bla*.2 could explain its decreasing prevalence seen when analysing all available WGS in the PubMLST database from 2010 onwards (Figure 4B). To account for any bias in sampling, we also examined the prevalence of p*bla*.1 and p*bla*.2 within a lineage (Ng_cgc$_{400}$ 29) which harbours both these variants. Between 2010 and 2020, there has been a shift from p*bla*.2 to p*bla*.1 in this lineage. In 2011, 55.6% of p*bla* sequences were p*bla*.2 and 33.3% p*bla*.1, while only p*bla*.1 was recovered from isolates of Ng_cgc$_{400}$ 29 after 2018 (Figure 4E).

As the observed changes in p*bla* variant prevalence could result from uneven sampling, we further assessed the relative success of p*bla* variants by their abundance within lineages; if a strain acquires a plasmid which offers an advantage it will undergo clonal expansion and outcompete other strains belonging to the same lineage but lacking the plasmid. Therefore, we examined the percentage of strains with p*bla* in the three largest lineages carrying each

p*bla* variant. p*bla*.1 is highly prevalent in lineages (50 - 60% of p*bla* carriage in major p*bla*.1 lineages, Table 1). In contrast, p*bla*.2 is only present at low frequency in lineages (<20%, Table 1), or is found in lineages with other variants. p*bla*.3 which expresses TEM-135 with no obvious fitness cost is found in a high percentage of strains in a lineage (39 - 84%).

Taken together, fitness costs imposed by p*bla*.2 are consistent with its low prevalence in the gonococcal population compared with p*bla*.1. p*bla*.3 with TEM-135, which arose from p*bla*.2, confers elevated penicillin resistance without fitness costs, and is associated with the success of a small group of related lineages.

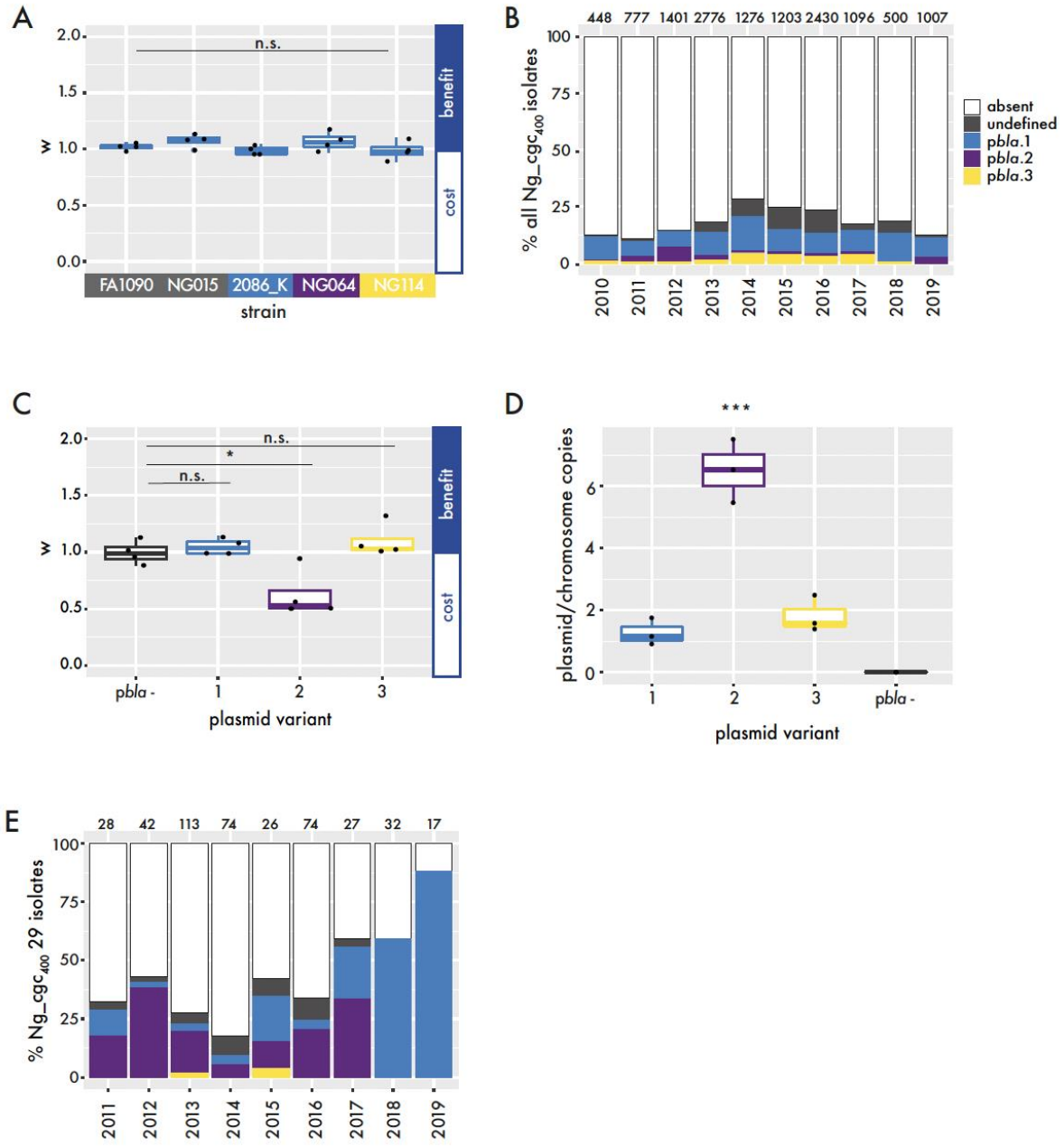

Figure 4: Impact of p*bla*-imposed fitness cost on its prevalence in the population. (A) Fitness cost (w) of p*bla*.1 in clinical isolates from different p*bla*-free (grey) or p*bla*-associated lineages (light blue, p*bla*.1-associated; purple, p*bla*.1/p*bla*.2-associated; yellow, p*bla*.3-associated). w>1 indicates a benefit, whereas w<1 signifies a cost of plasmid carriage. (B) Proportional p*bla* carriage in gonococcal isolates deposited on PubMLST between 2010 and 2019 (n=12,914 isolates). Colours show p*bla* variant carried and numbers above bars indicate the number of samples in the respective year. (C) Fitness cost of p*bla* variants in FA1090 isogenic strain background were assessed in four independent replicates (one-way ANOVA with Tukey multiple comparisons, n.s. p>0.05; * p<0.05). (D) Copy number of p*bla* in FA1090 isogenic strain background was assessed by ddPCR (one-way ANOVA with Tukey multiple comparisons; *** p<0.001). (E) p*bla* carriage in isolates from the p*bla*.1/p*bla*.2-associated Ng_cgc_{400} 29 between 2011 and 2019 (n=433 isolates). Bar colours indicate p*bla* variant and numbers above the bars specify the number of samples in the respective year.

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DISCUSSION

Plasmids are important vehicles for AMR, with resistance plasmids amongst the most diverse and mobile (42). Here, we investigated the origin of the relatively conserved beta-lactamase plasmid p*bla* which is largely found in the gonococcus, a WHO priority pathogen. Our analysis indicates that p*bla* was likely acquired by *N. gonorrhoeae* from another cause of STI, *H. ducreyi*. We describe the evolution of p*bla* since its emergence in *N. gonorrhoeae*, and its association with pConj. Gene loss and the appearance of novel TEM alleles influence the benefits and costs of p*bla* to the gonococcus. These traits are associated with the success and distribution of p*bla* variants within the gonococcal population.

In women, *N. gonorrhoeae* primarily causes cervicitis, while *H. ducreyi* causes ulcers at the vaginal entrance and cervix. In men, *N. gonorrhoeae* primarily causes urethritis and *H. ducreyi* mainly causes penile ulcers (43). Therefore, these species can occupy the same niche, providing ample opportunities for gene transfer. The presence of p*bla*.1 and p*bla*.2-like plasmids in *H. ducreyi* could indicate independent introductions of p*bla*.1 and p*bla*.2 into the gonococcus or independent emergence of p*bla*.1 in *H. ducreyi* and *N. gonorrhoeae*. Whilst we cannot reject either scenario, the independent emergence of p*bla*.1 in *H. ducreyi* and *N. gonorrhoeae* through the deletion of *repB*/NEIS2964 allele 2 and *repB*/NEIS2964 allele 3, respectively, is the most parsimonious explanation.

p*bla* is found relatively frequently in *N. gonorrhoeae* and *H. ducreyi*, which inhabit the genitourinary tract, but is seldom present in pathogens which inhabit the nasopharynx (*e.g. H. influenzae* and *N. meningitidis*); p*bla* was not detected in any non-invasive *Neisseria* spp. (41,158 isolates, 30 species). This could reflect the renal excretion of beta-lactams (44),

306 favouring p*bla* carriage for bacteria inhabiting the urogenital tract compared with other
307 sites. The meningococcal urethritis clade (NmUC) evolved from ST-11 *N. meningitidis* by
308 acquiring genetic elements from *N. gonorrhoeae* (45, 46). So far, p*bla* has not been reported
309 in NmUC. However, we previously found an ST-11 *N. meningitidis* isolate harbouring p*bla* (1).
310 This raises the possibility that NmUC might acquire p*bla*, which could provide an entry point
311 of this plasmid into the meningococcal population, and the emergence of beta-lactamase
312 producing *N. meningitidis*.
313 Plasmids can be successful in bacterial populations either by horizontal transmission and
314 their ability to spread into diverse lineages, or through vertical transmission through their
315 stable inheritance while being beneficial to their host. Our data indicates that the success of
316 p*bla* depends both on its own mobility and its association with pConj variants that can
317 effectively mediate its spread. Given the higher rates of pConj conjugation (>75%) compared
318 with p*bla* mobilisation (~1%), the spread of p*bla* into a lineage is likely to be accompanied by
319 pConj, maintaining the close association between these plasmids. Interesting, unlike many
320 other conjugative plasmids (47), pConj lacks entry exclusion, so p*bla* can enter bacteria
321 already containing pConj, allowing proliferation of bacteria with successful p*bla*/pConj pairs,
322 such as p*bla*.1/pConj.1, across the gonococcal population.
323
324 The most frequent and widespread p*bla* variant, p*bla*.1, is mobilised efficiently by common
325 pConj variants and does not impose fitness costs. p*bla*.2 is also mobile but imposes fitness
326 costs. Compared with p*bla*.1, p*bla*.2 has a second replication initiation protein and
327 additional origins of replication (*i.e. ori2* and *ori3*) (1, 48) which might increase copy number
328 and/or impose the fitness costs of p*bla*.2; plasmid Rep proteins can sequester host DNA
329 replication machinery (41), causing fitness costs. We found that p*bla*.2 is present in lower

prevalence in lineages than p*bla*.1, with evidence of a shift from p*bla*.2 to p*bla*.1 in a single lineage (Ng_cgc$_{400}$ 29) over time. Whilst the observed shift in p*bla* variants could reflect sampling bias, it is consistent with strains carrying p*bla*.2 being outcompeted by plasmid-free isolates or those with other p*bla* variants. Further evidence is provided by a longitudinal 10 year study of over 1,700 gonococcal isolates from a single city in China (Guangzhou), which reported a marked increase in p*bla*.1 (p*bla* Africa, from 18.4 to > 90% of isolates) with a concurrent fall in p*bla*.2 infections (from 81.6 to 7.6%) due to expansion of successful clones harbouring p*bla*.1 (49).

p*bla*.3-associated lineages have undergone clonal expansion indicating its successful adaptation to the gonococcus. Phylogenetic analysis indicates that TEM-135 originally arose in p*bla*.2. However, despite increased resistance levels conferred by TEM-135, the fitness cost of p*bla*.2 has likely undermined the success of TEM-135 in this p*bla* variant. We found that p*bla*.3 evolved from TEM-135 carrying p*bla*.2 through gene loss. This prevented the plasmid from being mobile, but with the trade-off of avoiding fitness costs. Consequently, the prevalence of p*bla*.3 in gonococci is not due to transfer between isolates, but through the expansion of p*bla*.3-carrying isolates, potentially through the increased beta-lactam resistance associated with TEM-135 and/or its association with an otherwise successful lineage. Further work is required to distinguish between these and other possibilities.
Since the emergence of gonococcal p*bla* in the 1970s, the evolutionary trajectory of this plasmid has been marked by its association with pConj variants that enable its spread through the population, the appearance of plasmid variants with minimal costs, and emergence of TEMs promoting higher resistance (*e.g.* TEM-135). A major concern is that the

ESBL-permissive M182T substitution in TEM-135 is already widespread in gonococci (1, 8), especially in p*bla*.3.

The intimate relationship between p*bla* and pConj also highlights the threat posed by increased use of tetracyclines, as already witnessed in LMICs where gonococci have remarkably high plasmid carriage (7, 50). Similarly, the implementation of Doxy-PEP will increase selection for the carriage of pConj in gonococci and will thence select the isolates/lineages harbouring the plasmid. A significant threat is posed by isolates carrying pConj as well as chromosomal mutations conferring cefotaxime resistance (51). The spread of these isolates, as well as p*bla* (which itself could become an ESBL-plasmid) have the potential to undermine the successful treatment of cases and their contacts with third-generation cephalosporins, the first-line antibiotics currently used to control gonococcal infection.

METHODS

Analysis of *Haemophilus* spp. and *Neisseria* spp. genomes

Tn*2*-carrying isolates of *Haemophilus* spp. and *Neisseria* spp. were identified querying Tn*2* (GenBank accession: LC091537.1) against *Haemophilus* (accessed 8/4/2025, 6,403 isolates, 12 species) and *Neisseria* (accessed 24/07/2024, 41,158 isolates, 33 species) sequences in PubMLST (23) (blastN word size: 11, scoring: reward: 2; penalty: -3; gap open: 5; gap extend: 2, sequence identity >99%, alignment length >50% query). p*bla*-like plasmids were confirmed by the presence of NEIS2960 (sequence identity >80%; alignment length >50%) and NEIS2358, and NEIS2961(1).

Plasmid variants

p*bla* and pConj were analysed in 15,529 gonococcal WGS on PubMLST (accessed 28/07/2022, Supplementary Table 4)(1, 23) with isolates from 1928-2022 and 66 countries. NEIS2220 indicates the presence of pConj, and variants were defined according to gene presence/absence and specific alleles of plasmid genes (8). p*bla* variants were typed using the Ng_p*bla*ST scheme (1). For the population structure, isolates were grouped into core genome clusters according to the allelic profile of 1,668 core genes(40); isolates were grouped with a cut-off of 400 allelic differences (Ng_cgc_{400}).

Phylogenetic analyses

A subset of 414 p*bla*-carrying isolates conserving the ratio of p*bla* variants (70% p*bla*.1, 14% p*bla*.2, 16% p*bla*.3, Supplementary Table 3) (1) was selected to investigate the phylogenetic relationship of p*bla* variants. This included all p*bla*-containing isolates pre-dating 2000 (n=35). Isolates between 2000 and 2022 (n=379) were randomly selected using the r sample function (1). Snippy v4.6.0 mapped plasmid reads to *H. ducreyi* DMC64 p*bla* (minimum coverage, 4 and base quality, 25). Multiple sequence alignments were generated with snippy-core v4.6.0/snippy-clean v4.6.0. Maximum likelihood trees were generated using RaxML-ng v1.2.2 (53) with 100 bootstrap replicates, rooted at *H. ducreyi* DMC64 p*bla*, and visualised with ape (54) and ggtree (55, 56).

Structure predictions

Analysis of NEIS2962 and RSF1010 MobC (GenBank accession: S96966.1) homodimers and NEIS2962 with p*bla oriT* (38) were performed using AlphaFold 3 (57) and PyMol v2.5.4 (58). Charge distributions were visualised with the Adaptive Poisson-Boltzmann Solver (APBS) electrostatics tool (59).

Bacterial strains/growth

Strains and plasmids used in this study are listed in Supplementary Tables 5 and 6, respectively. *E. coli* DH5α was grown on Luria-Bertani (LB) agar or in liquid LB shaking at 180 rpm. *N. gonorrhoeae* was grown on Gonococcal Base Media (GCB) agar plates or liquid media (GCBL) (60) supplemented with 1% Vitox (Oxoid) at 37°C in 5% CO_2. *H. ducreyi* was grown on chocolate agar plates supplemented with 1% IsoVitaleX at 35°C in 5% CO_2. Antibiotics were added as follows: for *E. coli*, carbenicillin 100 μg/ml; for *N. gonorrhoeae*,

carbenicillin 2.5 μg/ml; erythromycin 1 μg/ml; kanamycin 50 μg/ml, and tetracycline 2 μg/ml.

Characterisation of *H. ducreyi* plasmids

Genomic DNA was isolated from *H. ducreyi* by harvesting bacteria from plates and the DNeasy Blood/Tissue Kit (Qiagen) with the modifications that cells were incubated in lysis buffer with 20 mg/ml lysozyme at 37°C for 2 hours and then proteinase K overnight at 56°C. Plasmids were analysed by Sanger sequencing using primers listed in Supplementary Table 7. Sequence similarity of plasmid sequences was assessed with ClustalW with default parameters (61) and sequences were mapped onto *H. ducreyi* DMC64 p*bla* using Snapgene v6.1.1 (Insightful Science; available from snapgene.com) to investigate deletion sites.

Transformation of gonococci

For electroporation, bacteria grown on GCB agar were resuspended in PBS (Sigma), adjusted to $5x10^7$ CFU/ml then washed three times with 20% glycerol / 1% MOPS (Sigma); electroporation was performed with 2.5 kV, 200 Ω, 25 mF. Cells were recovered in 1 ml of GCBL with 2% Vitox and plated on GCB agar. Plates were incubated for 3 hours, cells collected, and then transferred to selective media.

Δ*pilD*::*ermC* and Δ*pilD*::*aph* constructs were transformed into *N. gonorrhoeae* as described previously (29, 31). In brief, 1 μg of DNA was spotted onto plates, allowed to dry, and bacteria streaked over the spots. Plates were incubated for 8 hours, then bacteria were transferred onto selective agar. Transformants confirmed by PCR/Sanger sequencing.

Plasmid modifications

All primers are listed in Supplementary Table 8. To generate p*bla*.1^{iso} and p*bla*.3^{iso}, p*bla*.2 was cut with *Hin*dIII-HF and *Pvu*II-HF (NEB), respectively. p*bla*.1^{iso} was amplified with primers TE18/19 and PrimeSTAR GXL polymerase (Takara Bio); Gibson assembly was performed with primers TE20/21. p*bla*.3^{iso} was amplified in two fragments with TE7/TE17 and TE9/TE16. Plasmids were assembled using Gibson Hifi (NEB) and transformed into *E. coli* DH5α.

Point mutations in *bla*TEM were introduced using the RAIR method (62). PCRs with primers (TE56/TE57, p*bla*.2 TEM-135; TE63/TE64, p*bla*.2$^{TEM\text{-}1\ P14S}$; TE65/TE66, p*bla*.3$^{TEM\text{-}1}$) were performed using Herculase II polymerase (Agilent), with p*bla*.2 or p*bla*.3 as template. Products were purified (Promega Wizard PCR Clean-up) and transformed into *E. coli* DH5α.

tetM^{+} pConj.7 was constructed by amplifying *tetM* from *N. gonorrhoeae* WHO N using primers TE34/TE35. Flanking regions were amplified with primers TE36/37 and TE38/39, then joined by Gibson assembly; the product was amplified with TE36/39, then introduced into *N. gonorrhoeae* NG028 by transformation. All constructs were confirmed by sequencing.

Conjugation and mobilisation assays

Donor (Δ*pilD*::*ermC*) and recipient (Δ*pilD*::*aph*) strains grown overnight were inoculated in 5 ml GCBL/1% Vitox at an OD_{600} of 0.1 and grown to mid-exponential phase (OD_{600} 0.6 - 0.8). The bacterial density was adjusted to 10^8 CFU/ml and donor and recipient strains mixed in a 10:1 ratio. Bacteria (5 μl) were spotted onto GCB agar and incubated for 6 hours at 37°C, 5% CO_2, harvested in 200 μl GCBL, then plated to GCB agar with antibiotics. Conjugation and mobilisation frequencies were defined as the number of transconjugants/recipients (n=3).

458 Plasmids used in the different assays are listed in Supplementary Table 6 together with the
459 WGS of the corresponding isolates available on PubMLST.
460
461 **Competition assays to determine fitness costs**
462 Plasmids were introduced into FA1090 Δ*pilD*::*ermC* and competed against FA1090 Δ*pilD*::*aph*
463 (n=4). Bacteria in PBS were adjusted to an OD_{600} 1, mixed 1:1, diluted to 10^5 CFU/ml and
464 added to 200 µl Fastidious Broth (63) then grown at 37°C, 5% CO_2, shaking at 180 rpm. After
465 24 hours, strains were enumerated by spotting on selective media. Fitness costs (w) were
466 calculated by:
467
468 $$w = \frac{ln(N_f/N_i)}{ln(N_{f,pbla}/N_{i,pbla})}$$
469
470 (w, relative fitness of p*bla*$^+$ *vs*. p*bla*$^-$ strains; $N_i$and N_f, p*bla*$^-$ strain at the beginning/end,
471 respectively; $N_{i,pbla}$and $N_{f,pbla}$, same for p*bla*$^+$ strain).
472
473 **Antibiotic susceptibility testing**
474 Penicillin G MICs were assessed using the broth microdilution method(64) in 96-well plates
475 with 2-fold Penicillin dilutions in water (50 µl); strains grown overnight on GCB agar were
476 resuspended in PBS (Sigma), then diluted in 2x FB/2% Vitox to 10^5 CFU/ml. Bacteria (50 µl)
477 were transferred into each well and incubated for 24 hours.
478
479

SDS page and Western blot analysis

Bacteria were grown to mid-exponential phase, added to an equal volume of 2x SDS-PAGE buffer, run on 12% SDS-polyacrylamide gels, and transferred to Protan nitrocellulose membranes (GE Healthcare) using the Trans-Blot Turbo System (Bio-Rad). Membranes were blocked in PBS/0.5% Tween-20/5% milk, washed thrice and incubated with the primary antibodies (Rabbit anti-RecA, Abcam, ab63797, 1:5,000; Mouse anti-TEM, Abcam, 8A5.A10, 1:1,000) for 2 hours. After washing, membranes were incubated with secondary antibodies (LI-COR Biosciences, 925-68071 IRDye® 680RD Goat anti-Rabbit IgG and 925-32210 IRDye® 800CW Goat anti-Mouse IgG) at a final dilution of 1:10,000 for 1 hour, washed, then imaged using LI-COR Biosciences.

Plasmid copy number

Copy number of *recA* and plasmid *tnpR* were quantified using the QX200 Droplet Digital PCR system (ddPCR, Bio-Rad) as described previously(65). ddPCR contained 1x EvaGreen super mix (Bio-Rad), and TE79/TE80 (*recA*, Supplementary Table 8) or TE81/TE82 (*tnpR*, Supplementary Table 8). After thermal cycling, data were analysed using the QX200 Droplet Reader with QuantaSoft software (Bio-Rad).

TEM mRNA expression

p*bla*.1^{TEM-1} carrying isolates were sub-cultured to mid-exponential phase in 5 ml GCBL/1% Vitox. Cells were harvested from 1 ml of culture and RNA was extracted using the Qiagen RNeasy Mini Kit together with the RNA protect Reagent (Qiagen, #76506) and on column RNase-free DNase I (Qiagen, #79254) treatment according to the manufacturer's instruction (protocol RY28). RNA was subsequently reverse transcribed to cDNA using the LunaScript RT

SuperMix Kit (NEB, #E3010). The cDNA was used in ddPCRs with primers TE79/80 (*recA*) and TE97/98 (*bla*TEM, Supplementary Table 8). The no-RT and no template reactions served as negative controls.

Statistics and data analysis

Data analysis was performed in R version 4.1.1 using base R and the tidyverse package(66). Plots were generated with ggplot2 (67). A p value <0.05 was considered statistically significant.

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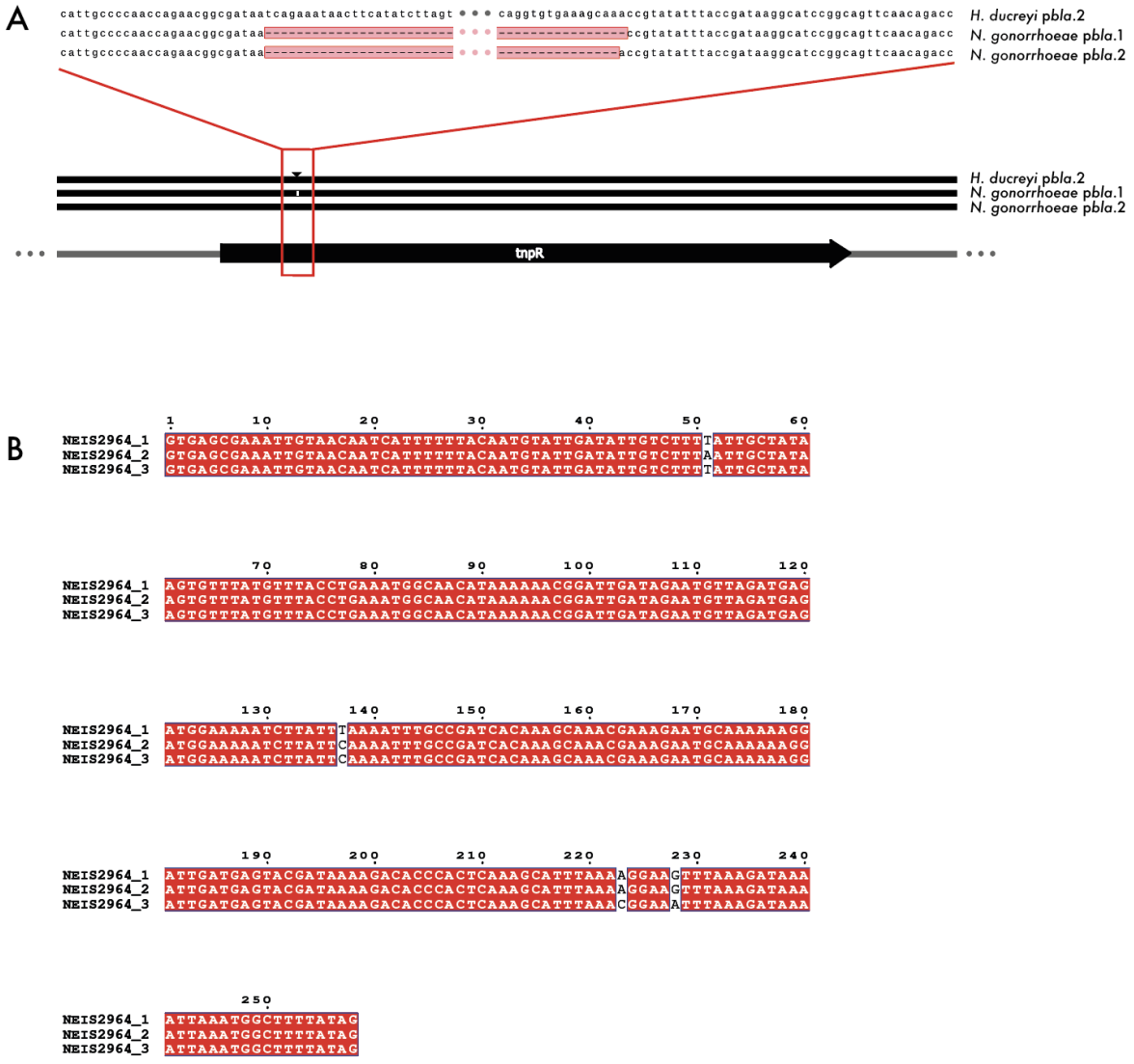
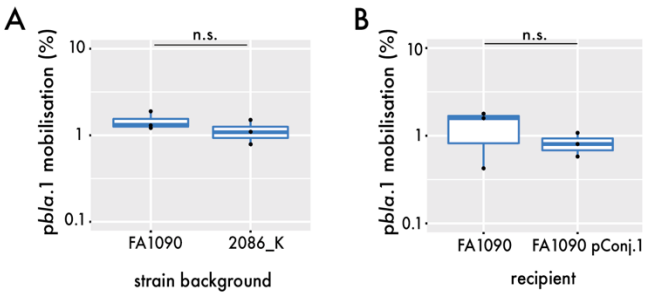
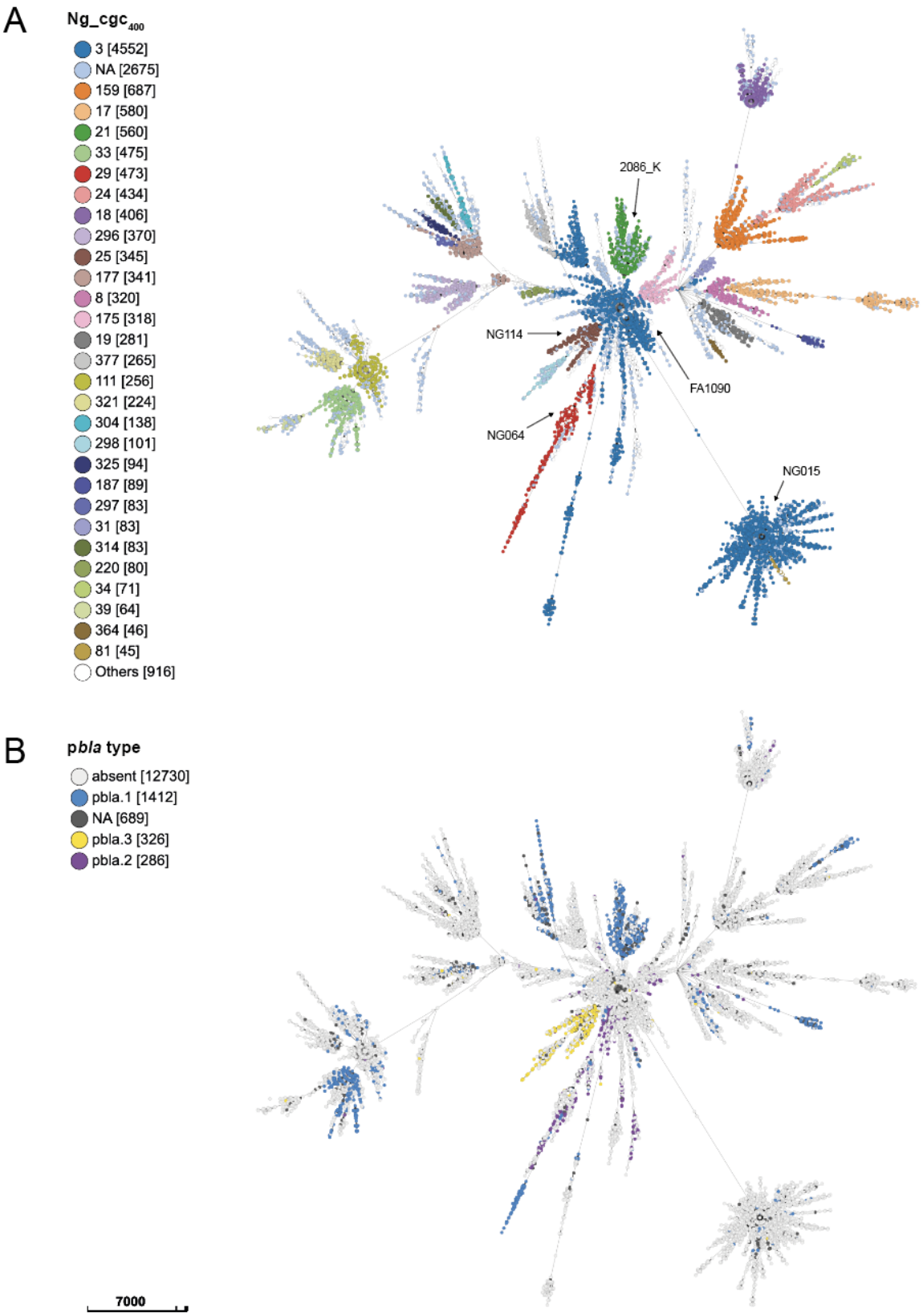
SUPPLEMENTARY FIGURES



Supplementary Figure 1: (A) Analysis of the Tn*2* deletion region: the truncation affecting *tnpR* in gonococcal p*bla*.1 and p*bla*.2 differ by a single nucleotide. Schematic representation of *tnpR* sequences of gonococcal and *H. ducreyi* p*bla*s is shown on the bottom with the SNP displayed as a white line and the site of the Tn*2* truncation in relation to *H. ducreyi* p*bla* indicated with a triangle. Displayed above is the nucleotide alignment of gonococcal p*bla* to *H. ducreyi* DMC64 p*bla*.2. (B) Sequence alignment of NEIS2964 alleles -1 to -3) with SNPs highlighted in white.



Supplementary Figure 2: p*bla*.1 mobilisation by pConj.1 in isogenic matings. (A) p*bla.*1 mobilisation in the *N. gonorrhoeae* strains FA1090 and 2086_K (Welch two-sample t-test, p = 0.66). (B) Mobilisation rates of p*bla*.1 into FA1090 with or without pConj (Welch two-sample t-test, p = 0.87).



Supplementary Figure 3: Minimum spanning trees of *N. gonorrhoeae* clustered by core genome allelic differences with distribution of p*bla* variants. Individual dots represent isolates that are coloured according to Ng_cgc$_{400}$ (A) or p*bla* variant carried (B). Strains used in this study are indicated in panel A.

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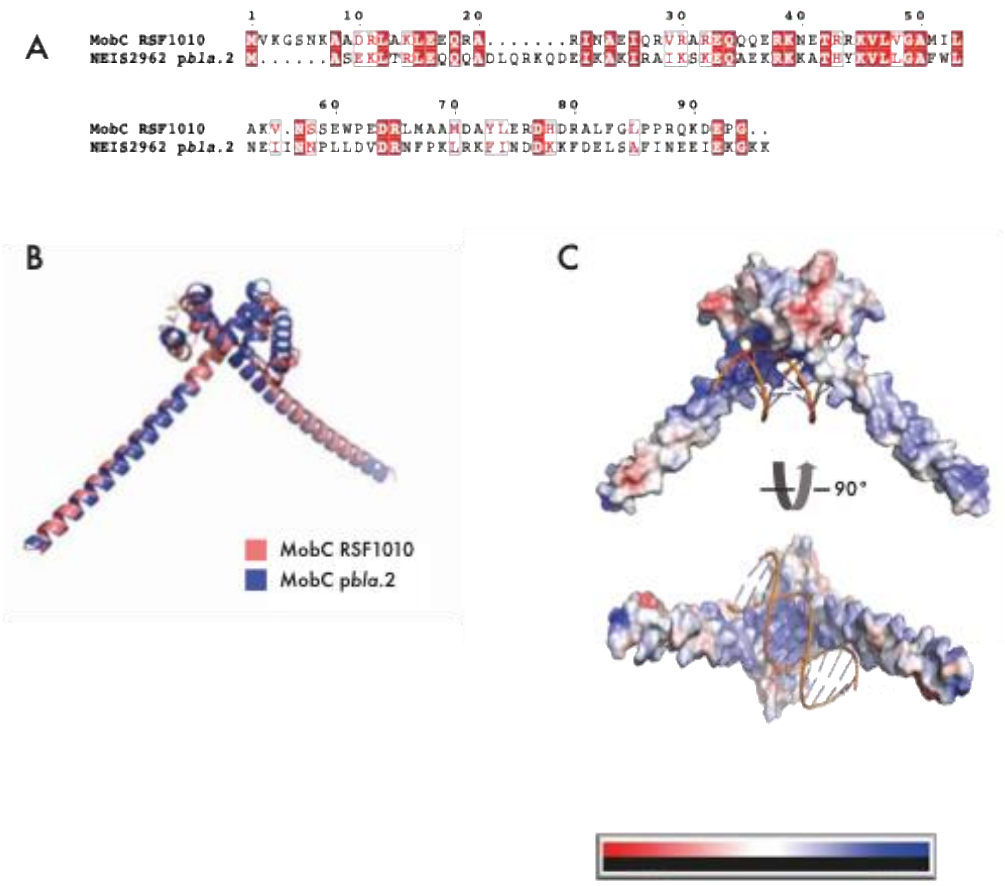

Supplementary Figure 4: (A) Alignment of MobC from *E. coli* plasmid RSF1010 (Genbank accession: S96966.1) and NEIS2962 from p*bla*.2 (Genbank accession: NZ_LT591911). Amino acid sequences were aligned with COBALT(2) and the alignment visualised with Esprit (3). Identical residues are shown in white on red background, residues with a similarity score >0.7 are framed in blue and the remaining residues are shown in black. (B) Superimposed AlphaFold structure prediction of MobC from the *E. coli* plasmid RSF1010 (salmon, Genbank accession: S96966.1) and NEIS2962 (blue) dimers (Match Align: 677.7, RMSD: 0.775Å) (C) Electrostatics prediction of NEIS2962 homodimer with *oriT* sequence using the Adaptive Poisson Boltzman Solver Electrostatics Plugin. Negatively and positively charged regions are shown in red and blue, respectively.

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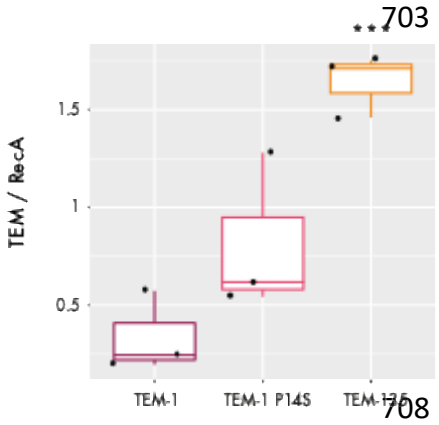

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Supplementary Figure 5: Cellular levels of different TEM variants in an isogenic FA1090 background. TEM/RecA ratios of whole cell lysates were visualised by Western blot analysis and quantified with the LI-COR system (one-way ANOVA with Tukey multiple comparisons, n.s. p>0.05; *** p<0.001).

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S Figure 4


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S Figure 1


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Figure 2

Figure 3

Figure 4

Figure 1

Origin, evolution, and success of p*bla*, the gonococcal beta-lactamase plasmid, and implications for public health

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ABSTRACT

Neisseria gonorrhoeae **is a leading cause of sexually transmitted infection (STI) and a priority AMR pathogen. Two narrow host range plasmids, p*bla* and pConj, have contributed to ending penicillin and tetracycline~~/doxycycline~~ therapy, respectively, and undermine current prevention strategies including doxycycline post-exposure prophylaxis (Doxy-PEP). Here, we investigated the origin and evolution of the beta-lactamase plasmid, p*bla*. We demonstrate that p*bla* was likely acquired by the gonococcus ~~on at least two occasions~~ from *Haemophilus ducreyi*, and describe the subsequent evolutionary pathways taken by the three major p*bla* variants. We show that the ability of pConj to spread p*bla* promotes their co-occurrence in the gonococcal population and the spread of p*bla*. Changes that mitigate fitness costs of p*bla* and the emergence of TEM beta-lactamases that~~which~~ confer increased resistance have contributed to the success of p*bla*. In particular, TEM-135, which has arisen in certain p*bla* variants, increases resistance to beta-lactams and only requires one amino acid change to become an extended spectrum beta-lactamase (ESBL). The evolution of p*bla* underscores the threat of plasmid-mediated resistance to current therapeutic and preventive strategies against gonococcal infection. Given the close relationship between p*bla* and pConj, widespread use of Doxy-PEP is likely to promote spread of both plasmids, strains which carry pConj and are resistant against third generation cephalosporins, and the emergence of plasmid-mediated ESBL in the gonococcus, with significant public health consequences.**

AUTHOR SUMMARY

Neisseria gonorrhoeae is a threat to sexual and reproductive health. With no available vaccine against gonococcal disease, control of infection depends on having effective antimicrobials for the treatment of cases and their contacts. However, strains of *N. gonorrhoeae* have acquired resistance to all classes of antibiotics, including third generation cephalosporins, the mainstay of treatment for gonococcal disease. The gonococcus can carry two resistance plasmids, the conjugative plasmid pConj and beta-lactamase plasmid p*bla*, which have rendered treatment with tetracycline/doxycycline and penicillin ineffective, respectively. Here, we investigated the origin and evolution of p*bla,* and the molecular basis of its success in the gonococcal population. We show that p*bla* was likely acquired ~~on at least two occasions~~ from *Haemophilus ducreyi* and has adapted to the gonococcus both through gene loss that reduces fitness costs, and through mutations that increase resistance and favour the development of an extended spectrum beta-lactamase. Our findings show that the ability of pConj to mobilise p*bla* promotes their co-occurrence, highlighting the potential to co-select for p*bla* by favouring pConj-containing strains through doxycycline use. Our results highlight the continuing adaptation of p*bla* to its host, which could undermine treatment options in the future.

INTRODUCTION

Neisseria gonorrhoeae causes ~80 million cases of sexually transmitted infection (STIs) annually (4) and is a WHO priority pathogen due to its extensive antimicrobial resistance (AMR) (5). *N. gonorrhoeae* has two resistance plasmids, pConj and p*bla*, which these plasmids contributed to the cessation of tetracycline and penicillin therapy, respectively. Furthermore, pConj carrying *tetM* confers resistance to doxycycline as well as tetracycline, so has undermined the ability of doxycycline post-exposure prophylaxis (Doxy-PEP) to prevent gonococcal infection (6, 7). pConj and p*bla* are highly prevalent in low and middle-income countries (LMICs) where syndromic treatment of STIs with doxycycline has been recommended (1, 7, 8). Therefore, it is important to understand the factors driving the success of these plasmids in gonococcal populations.

pConj is a 39-42 kb conjugative plasmid, that can confer high-level tetracycline/doxycycline resistance (7, 9), and can be categorised into seven variants (8). p*bla* emerged in the gonococcus in the 1970s and encodes a TEM beta-lactamase conferring penicillin resistance (10, 11). p*bla* TEM beta-lactamases require a few changes amino acid changes to become an extended-spectrum beta-lactamase (ESBL) (12), which would render third-generation cephalosporins, the current first-line treatment, ineffective (13). p*bla* is usually closely associated with pConj which can mobilise p*bla* (8, 14); around 85% of strains that harbour p*bla* also contain pConj.

There are three main p*bla* variants, characterised by the presence/absence of certain genes (1). The 7.4 kb p*bla*.2 (also referred to as p*bla* Asia) has been considered the ancestral

plasmid (15). p*bla*.1 (5.6 kb, p*bla* Africa) is the commonest variant and has a deletion in the replication region, while p*bla*.3 (5.1 kb, p*bla* Rio/Toronto) lacks the region implicated in p*bla* mobilisation (1, 15). Variants of p*bla* are associated with certain pConj variants and TEM alleles (1). p*bla*.1 mostly carries TEM-1 or TEM-1_{P14S}, while p*bla*.3 is associated with TEM-135; p*bla*.2 carries TEM-1 or TEM-135. Importantly, the M182T substitution in TEM-135 is a 'stepping stone' mutation before the enzyme becomes an ESBL (12, 16).

Here, we investigated the origin, evolution and characteristics of the three p*bla* variants. We demonstrate that the spread and distribution of p*bla* in gonococci results from the dynamics ~~interplay~~ of its association with pConj, fitness costs, and resistance levels. p*bla* is found with mobile pConj variants~~,~~ and has evolved to avoid fitness costs and confer higher resistance to beta-lactams. Our results underline the threats posed by p*bla* and pConj, particularly if their spread and prevalence in the gonococcal population is promoted by the widespread implementation of Doxy-PEP.

RESULTS

p*bla* has been acquired ~~at least twice~~ by the gonococcus from *Haemophilus*

Haemophilus spp. are known to harbour~~s~~ plasmids related to p*bla* (17-22); ~~,~~ however, without nucleotide sequence data, these early studies could not ~~precisely~~ characterise the precise relationship between *Haemophilus* and *Neisseria* beta-lactamase plasmids. Therefore, we interrogated *Haemophilus* whole genome sequences (WGS) in the PubMLST database (23) (6,896~~4,620~~ isolates, 12 species, Supplementary Table 1) for p*bla*. We searched for Tn*2* as p*bla* TEM-1b is located on this transposon (24, 25), and confirmed the presence of p*bla* replicons by searching for NEIS2960, NEIS2358 (*repA*), and NEIS2961 (*mobA*) (1). Tn*2* is present in 12.5~~10.2~~% of *Haemophilus influenzae* (~~4,337~~6,403 isolates) and 19.3 % *Haemphilus~~,~~ parainfluenzae* (269 isolates), while p*bla*-like replicons were detected in 0.3 % of *H. influenzae* and 1.5% *H. parainfluenzae* isolates. However, Tn*2* and p*bla* sequences only co-occurred ~~with a p*bla* like plasmid only co-occurring~~ in two isolates of *H. influenzae* (PubMLST id~~s~~: 23482 and 33361) and *H. parainfluenzae* (PubMLST ids: 16289 and 34872).~~.~~

In contrast, seven of 31 (22.6%)~~ of~~ *H. ducreyi* isolates (~~7/31,~~ Supplementary Table 2) harbour TEM-1 containing p*bla*-like plasmids. *H. ducreyi* strains can be divided into two clades/classes (26), and a p*bla.*1~~-~~like plasmid in Class I isolate and a p*bla.*2-like plasmid~~s~~ in ~~Class I~~ a ~~and~~ Class II isolates. The sequences of plasmids from *H. ducreyi* HD183 (Class I, 9.1 kb) and DMC64 (Class II, 10.9 kb) (27) were aligned to gonococcal p*bla*.1 and p*bla*.2, and were found to be highly similar (> 83% , Figure 1A). p*bla*.1 and the 9.1 kb *H. ducreyi* plasmid carry *repB* and one copy of NEIS2964. Both the 10.9 kb *H. ducreyi* plasmid and gonococcal p*bla*.2 carry two *rep* alleles~~,~~ *repA* and *repB,* as well as two copies of NEIS2964. The major

difference is that Tn*2* is intact in the *H. ducreyi* plasmids while gonococcal p*bla* lack *tnpA* and have a truncated *tnpR.* These findings are consistent with p*bla* transfer from *H. ducreyi* into the gonococcus with the event associated with truncation of Tn*2*.

Two alternative scenarios can explain the occurrence of two distinct p*bla* variants in both *H. ducreyi* and *N. gonorrhoeae*: (1) independent introductions of p*bla*.1 and p*bla*.2, with both introductions associated with a truncation of Tn*2*, or (2) introduction of p*bla*.2 into the gonococcus with p*bla*.1 emerging independently in *H. ducreyi* and *N. gonorrhoeae* through the deletion of *repB* and one copy of NEIS2964. Comparison of the Tn*2* deletion site in gonococcal p*bla* variants demonstrates that the plasmids carry distinct *tnpR* alleles (allele 3 and 2 for p*bla*.1 and p*bla*.2, respectively), as their truncations differ by a single nucleotide (Supplementary Figure 1A), supporting the hypothesis of two independent Tn*2* truncations/introductions. We next examined the p*bla*.1 deletion site (spanning *repB* and one copy of NEIS2964). *H. ducreyi* p*bla*.2 carries two copies of NEIS2964 (alleles 2 and 3) which differ by three nucleotides (Supplementary Figure 1B). Gonococcal p*bla*.2 carries alleles 1 and 2. *H. ducreyi* p*bla*.1 carries allele 3, whereas gonococcal p*bla*.1 is associated with allele 2, suggesting p*bla*.1 arose independently in *H. ducreyi* and *N. gonorrhoeae*. Therefore, analysis of Tn*2* truncations and p*bla* deletions is inconclusive about whether a ~~the~~ single or ~~/~~multiple introductions of p*bla* occurred into the gonococcus.

To investigate the evolutionary relationships between gonococcal p*bla* variants further, we examined a subset of p*bla* (414 of 2,758, Supplementary Table 3~~2~~) (1) with the 10.9 kb *H. ducreyi* p*bla* as reference; plasmids were from 1979-2022 with the same proportion of

variants as the whole population (*i.e.* 70% p*bla*.1, 14% p*bla*.2, 16% p*bla*.3) (1). A m~~M~~aximum likelihood phylogeny placed ~~distinguished~~ p*bla* variants into distinct clades, with p*bla*.1 split from the other variants. While *H. ducreyi* p*bla* carry TEM-1, TEM-1_{P14S} arose in p*bla*.1 while TEM-135 arose in p*bla*.2, with p*bla*.3 emerging from TEM-135 carrying p*bla*.2 (Figure 1B).

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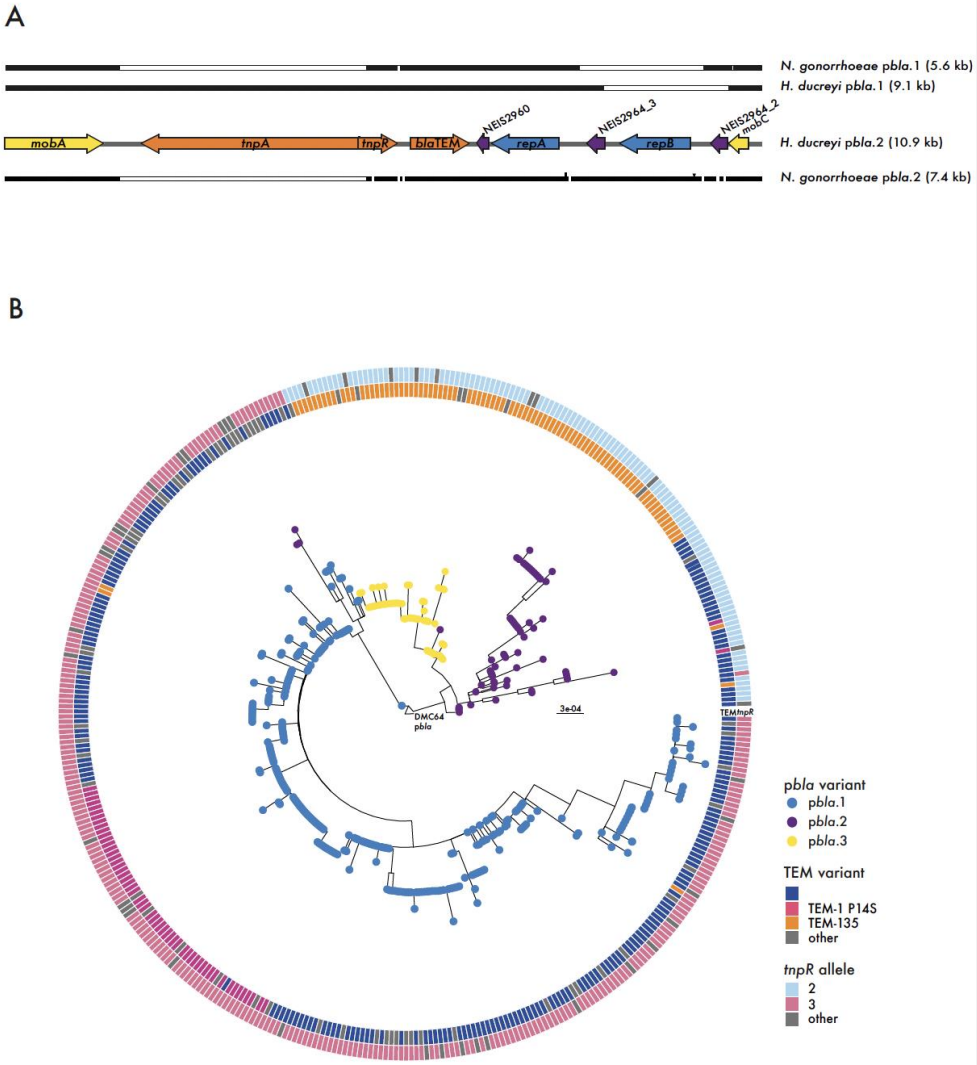

Figure 1: Evolutionary relationships between p*bla* variants. (A) Schematic representation of alignment of gonococcal and *H. ducreyi pbla* variants to *H. ducreyi pbla*.2. Aligned regions are represented as black bars, with deletion regions and ~~SNPs~~ nucleotide polymorphisms indicated in white within. Insertions are shown as black lines above the bars. ORFs on *H. ducreyi pbla*.2 are coloured according to gene function; yellow, mobilisation proteins; orange, Tn*2*-derived genes including *bla*TEM; light blue, replication initiation proteins; ~~dark green~~purple, ~~undefined/~~other gene function. (B) Maximum likelihood tree of 414 gonococcal p*bla* sequences with tips coloured according to p*bla* variant; the tree was rooted at *H. ducreyi* p*bla*.2. Circles indicate the *tnpR* allele and the TEM variant carried.

p*bla* is associated with pConj variants that promote its spread

To understand the association between p*bla* and pConj, we examined the ability of different pConj variants to transfer p*bla*. Initially, matings were performed between isogenic strains (FA1090 or 2086_K) to eliminate any barrier to horizontal gene transfer between unrelated strains (28). Additionally, Δ*pilD* donors and recipients were constructed to block transformation (29-31), and the transfer p*bla*.1 by pConj.1, the commonest combination of these plasmids (1), was measured. p*bla*.1 was mobilised ~~by~~ at a frequency of ~1% transconjugants/recipient for FA1090 and 2086_K (Supplementary Figure 1), while no p*bla* transfer was detected in the absence of pConj.

Conjugative plasmids can block the acquisition of other plasmids by expressing entry exclusion proteins (32). E~~;~~ entry exclusion could impede p*bla* mobilisation, as the initial transfer of pConj during conjugation could block subsequent acquisition of p*bla*. pConj encodes a predicted lipoprotein annotated as TrbK entry exclusion protein (33), with 21% amino acid similarity with *Agrobacterium fabrum* Ti plasmid TrbK (locus tag: ATU_RS23180, Genbank: NC_003065.3). To examine whether pConj in a recipient can impair the transfer of p*bla*, we compared the mobilisation rates of p*bla* into pConj-free and pConj-harbouring recipients. There was no difference in p*bla* transfer into pConj-free (1.1%) and pConj-containing recipients (0.9%, Welch two-sample t-test, p = 0.87, Supplementary Figure 1), indicating that surface exclusion is unlikely to limit p*bla* spread.

We next evaluated p*bla* mobilisation by pConj variants that are frequently associated with p*bla* (*i.e.* pConj 1, 3, and 4, Figure 2A) and by variants that are infrequently associated with p*bla* (*i.e.* pConj.2 and 7). The conjugation frequencies of pConj.1, 3 and 4 were >79% (Figure

2B) but several orders of magnitude lower for pConj.2 and pConj.7 which are not associated with p*bla* (Figure 2A). T ~~Of note, t~~he rate of p*bla* mobilisation correlated with ~~mirrored~~ conjugation frequencies (Figure 2B), with p*bla* transfer by pConj.2 undetectable in our assays (limit of detection, L.O.D. = 0.001%). Taken together, results indicate that p*bla* is associated with pConj variants that mobilise it efficiently~~,~~ and promote its spread in the gonococcal population.

The immobility of p*bla*.3 is reflected in its restricted distribution

There are conflicting data about the mobility of p*bla*.3 (34-36). Therefore, we assessed the mobilisation of wild-type p*bla* variants by pConj.1. Results indicat~~demonstrat~~e that wild-type p*bla*.1 and p*bla*.2 we~~a~~re mobilised efficiently (transfer rate, ~ 1%), while p*bla*.3 mobilisation was not detected (Figure 2C). To assess whether p*bla* variant deletions are responsible for these differences, we introduced variant-specific deletions into p*bla*.2, generating the isogenic plasmids p*bla*.1^{iso} and p*bla*.3^{iso}. Mobilisation of the isogenic plasmids did not differ from wild-type plasmids (p = 0.82, Figure 2C), indicating the 2.3 kb deletion in p*bla*.3 compared with p*bla*.2 is responsible for the lack of p*bla*.3 transfer. T ~~Of note, t~~he immobility of p*bla*.3 is evident from its restricted distribution in three related lineages, whilst p*bla*.1 and p*bla*.2 are found across the gonococcal population (Supplementary Figure 3~~2~~) (1).

~~Next, we examined the genes responsible for the immobility of p*bla*.3 by generating p*bla*.2 mutants lacking genes absent in p*bla*.3.~~ Mobilisable plasmids deploy diverse strategies to exploit the conjugative machinery of co-existing conjugative plasmids (37). Some m~~M~~obilisable plasmids encode their own relaxase which recognises and nicks their origin of transfer (*oriT*) and then guides the plasmid DNA through the Type 4 secretion system

encoded by the conjugative plasmid. Alternatively, the *oriT* of mobilizable plasmids can be recognised by the relaxase encoded by a conjugative plasmid~~s~~. Therefore, we next examined the genes responsible for the immobility of p*bla*.3 by generating isogenic p*bla*.2 mutants lacking genes absent in p*bla*.3, and assessing their ability to be mobilised between *pilD* mutants of FA1090 by pConj1. The p*bla*.3-characteristic deletion spans *mobA* (NEIS2961, encoding the relaxase (38)), *mobC* (NEIS2962) and NEIS2964. Deletion of *mobA* abolished p*bla* transfer (Figure 2D), indicating that p*bla* mobilisation depends on its own relaxase and not the pConj relaxase. Removal of NEIS2962 significantly reduced p*bla* mobilisation ($p = 0.01$, Figure 2D). NEIS2962 is related to MobC from *E. coli* plasmid RSF1010, which unwinds DNA at the *oriT* (39). NEIS2962 homodimers are structurally related to MobC and predicted to recognise the p*bla* *oriT* but not a scrambled *oriT* sequence (Supplementary Figure 4~~3~~). Deletion of both copies of NEIS2964 did not impact p*bla*.2 transfer.

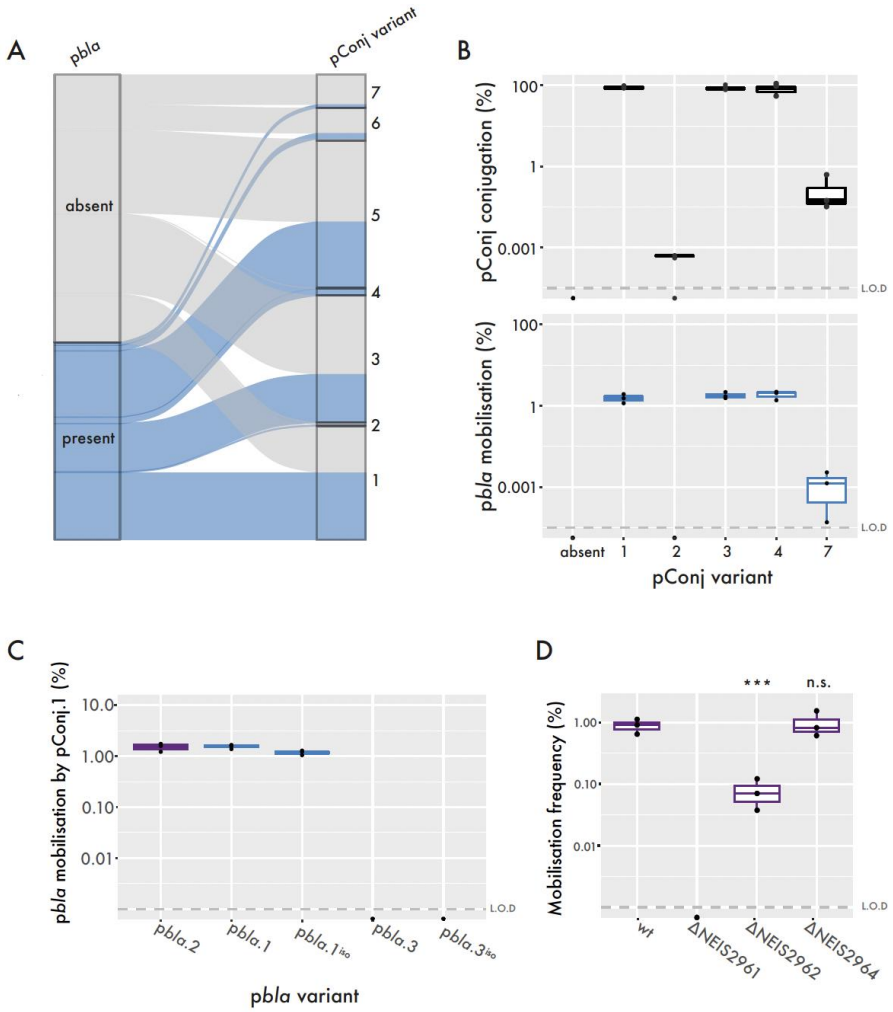

Figure 2: p*bla* mobilisation by pConj. (A) Sankey plot of pConj carrying isolates (n = 4,883 isolates) (1), displaying the presence of p*bla* (left) and co-occurrence of p*bla* with individual pConj variants (right). (B) Conjugation rates of pConj variants (top) and the mobilisation rates of co-located p*bla*.1 (bottom). The limit of detection (L.O.D.) is indicated as a dashed line. (C) Mobilisation rates of wild type and isogenic p*bla* variants (p*bla*^{iso}) by pConj.1. (D) The impact of single *mob* gene knockouts in p*bla*.2 on p*bla* mobilisation frequencies. All assays consist of three individual repeats and were analysed by one-way ANOVA with Tukey multiple comparisons; n.s. p>0.05, *** p<0.001.

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TEM-135 confers increased penicillin resistance

If a plasmid is beneficial to its host, one would expect that plasmid carrying isolates to be highly prevalent in a lineage, due to their competitive advantage of isolates without the plasmid. Therefore, to assess the prevalence of p*bla* in certain lineages, we resolved the gonococcal population structure by clustering isolates according to allelic differences in loci that are core to the gonococcus (40), and defined *N. gonorrhoeae* core genome clusters (Ng_cgc$_{400}$) with a cut-off of 400 allelic differences. Although p*bla*.3 is immobile, it is highly prevalent in Ng_cgc$_{400}$s 25 and 298 with 56.8 and 39.6% of isolates in these lineages carrying p*bla*, respectively, Table 1, Supplementary Figure 3~~2~~) (1). This suggests p*bla*.3, which carries TEM-135, confers a benefit to the gonococcus that has led to the clonal expansion of isolates containing this p*bla* variant. ~~Therefore~~To test this, we measured the penicillin MICs conferred by p*bla* variants. Whilst TEM-1 carrying p*bla*.1 and 2 conferred MICs of 8 µg/ml, p*bla*.3 with TEM-135 conferred a significantly higher MIC (32 µg/ml, p=0.003, Figure 3A). To establish whether the TEM variant determines resistance levels, we changed p*bla*.3 TEM-135 into TEM-1 by introducing a T182M substitution. This substitution reduced the MIC conferred by p*bla*.3 to levels of p*bla*.1 and p*bla*.2, demonstrating that TEM-135 confers elevated MICs (Figure 3B). We also compared resistance conferred by TEM-1, TEM-1$_{P14S}$ and TEM-135 (which together account for >95% of gonococcal TEMs (1)) expressed by p*bla*.2. Again, TEM-135 significantly increased MICs (128 µg/ml *vs.* 8 µg/ml with TEM-1, p<0.001, Figure 3C).

To ~~understand~~ explore the basis for the different MICs, we assessed cellular TEM levels. Levels of mature TEM-135 (29 kDa) were significantly higher than TEM-1 or TEM-1$_{P14S}$ (Figure 3D, Supplementary Figure 5~~4~~), consistent with increased stability of TEM-135 (16). In

conclusion, the appearance of TEM-135, particularly associated with p*bla*.3, provides a significant benefit to the gonococcus by enhancing resistance against beta-lactams, with MICs correlating with cellular TEM levels.

Table 1: The percentage of isolates carrying p*bla* in three largest Ng_cgc400 that carry each variant.

Ng_cgc$_{400}$	Number of isolates	p*bla* variant(s)	p*bla* carriage (%)
21	574	1	64.3
33	476	1	51.9
187	94	1	62.8
3	4545	1 / 2 / 3	1.2 / 0.5 / 0.3
29	473	1 / 2	14 / 18
175	318	2	8.5
25	346	3	56.8
298	101	3	39.6
391	31	3	87.1

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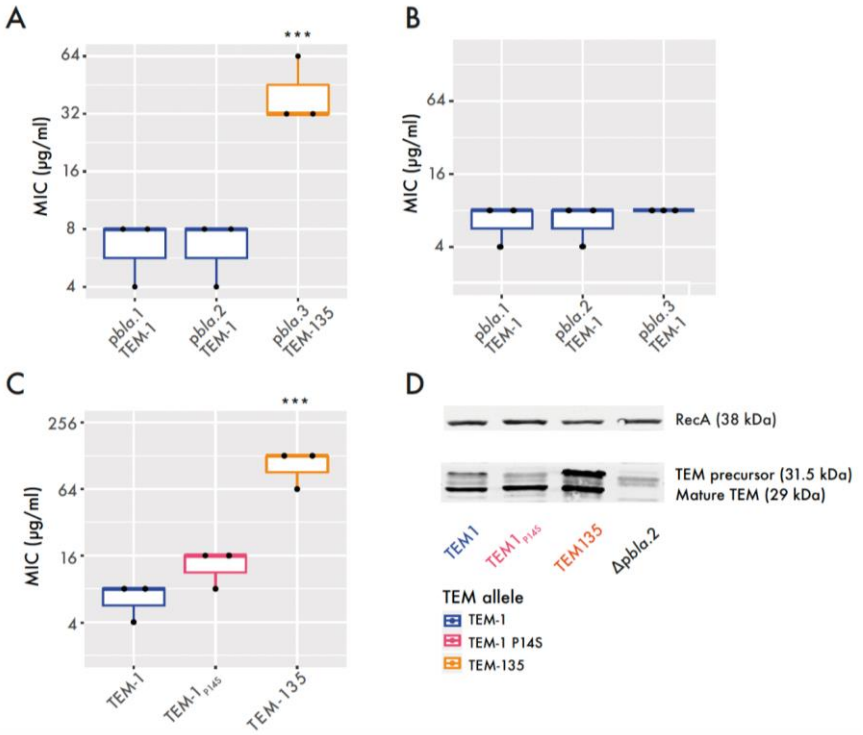

Figure 3: TEM-135 increases the MIC. (A) Penicillin G MICs of p*bla* variants in FA1090 isogenic strain background (one-way ANOVA on $\log_2$-transformed MIC values with Tukey multiple comparisons of means; *** p<0.001). (B) MICs of TEM-1 in different p*bla* variant backbones. (C) MICs of different TEM variants in p*bla*.2 backbone (one-way ANOVA on $\log_2$-transformed MIC values; *** p<0.001). (D) Cellular levels of TEM variants were assessed by Western blot analysis. The image is representative of three biological repeats.

Successful p*bla* variants have evolved with reduced fitness costs

Plasmids often impose fitness costs, disadvantaging isolates ~~which~~ that carry plasmids (41). We therefore assessed the fitness costs of p*bla* by introducing p*bla*.1 into isolates from a range of lineages (Supplementary Figure 3)~~,~~ and competing plasmid-carrying *vs.* plasmid-free strains over 24 hrs. p*bla*.1 had no detectable fitness cost in any isolate (Figure 4A), consistent with its continued prevalence in the gonococcus (Figure 4B). We also compared the fitness costs of the three p*bla* variants in wild-type FA1090. In contrast to p*bla*.1 and p*bla*.3 which impose no fitness cost, p*bla*.2 inflicts a significant fitness cost which was evident within 24 hrs (Figure 4C). This is associated with a higher copy number for p*bla*.2 (>6 copies/chromosome) than the other variants (1-2 copies/chromosome, Figure 4D).

The fitness costs of p*bla*.2 could explain its decreasing prevalence seen when analysing all available WGS in the PubMLST database from 2010 onwards (Figure 4B). To account for any ~~potential~~ bias in sampling, we also examined the prevalence of p*bla*.1 and p*bla*.2 within a lineage (Ng_cgc$_{400}$ 29) which harbours both these variants. Between 2010 and 2020, there has been a shift from p*bla*.2 to p*bla*.1 in this lineage. In 2011, 55.6% of p*bla* sequences were p*bla*.2 and 33.3% p*bla*.1, while only p*bla*.1 was recovered from isolates of Ng_cgc$_{400}$ 29 after 2018 (Figure 4E).

As the observed changes in p*bla* variant prevalence could result from uneven sampling, we further assessed the relative success of p*bla* variants by their abundance within lineages~~Further evidence of the relative success of each p*bla* variant can be revealed by their abundance within a lineage~~; if a strain acquires a plasmid which offers an advantage it will undergo clonal expansion and outcompete other strains belonging to the same lineage

but lacking the plasmid. Therefore, we examined the percentage of strains with p*bla* in the three largest lineages carrying each p*bla* variant. p*bla*.1 is highly prevalent in lineages (50 - 60% of p*bla* carriage in major p*bla*.1 lineages, Table 1). In contrast, p*bla*.2 is only present at low frequency in lineages (<20%, Table 1), or is found in lineages with other variants. p*bla*.3 which expresses TEM-135 with no obvious fitness cost is found in a high percentage of strains in a lineage (39 - 84%).

Taken together, fitness costs imposed by p*bla*.2 are consistent with its low prevalence in the gonococcal population compared with p*bla*.1. p*bla*.3 with TEM-135, which arose from p*bla*.2, confers elevated penicillin resistance without fitness costs, and is associated with the success of a small group of related lineages.

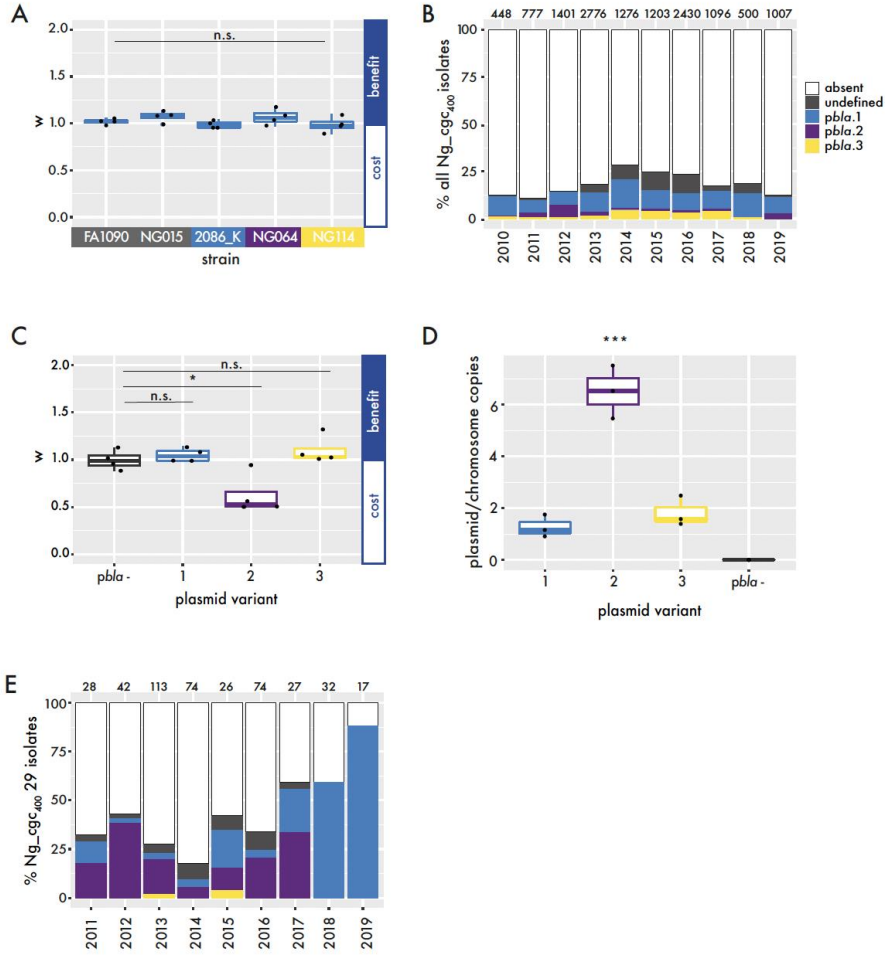

Figure 4: Impact of p*bla*-imposed fitness cost on its prevalence in the population. (A) Fitness cost (w) of p*bla*.1 in clinical isolates from different p*bla*-free (grey) or p*bla*-associated lineages (~~turquoise~~light blue, p*bla*.1-associated; purple~~ dark green~~, p*bla*.1/p*bla*.2-associated; yellow, p*bla*.3-associated). w>1 indicates a benefit, whereas w<1 signifies a cost of plasmid carriage. (B) Proportional p*bla* carriage in gonococcal isolates deposited on PubMLST between 2010 and 2019 (n=12,914 isolates). Colours show p*bla* variant carried and numbers above bars indicate the number of samples in the respective year. (C) Fitness cost of p*bla* variants in FA1090 isogenic strain background were assessed in four independent replicates (one-way ANOVA with Tukey multiple comparisons, n.s. p>0.05; * p<0.05). (D) Copy number of p*bla* in FA1090 isogenic strain background was assessed by ddPCR (one-way ANOVA with Tukey multiple comparisons; *** p<0.001). (E) p*bla* carriage in isolates from the p*bla*.1/p*bla*.2-associated Ng_cgc$_{400}$ 29 between 2011 and 2019 (n=433 isolates). Bar colours indicate p*bla* variant and numbers above the bars specify the number of samples in the respective year.

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DISCUSSION

Plasmids are important vehicles for AMR, with resistance plasmids amongst the most diverse and mobile (42). Here, we investigated the origin of the relatively conserved beta-lactamase plasmid p*bla* which is largely found in the gonococcus, a WHO priority pathogen. Our analysis indicates that p*bla* was likely acquired by *N. gonorrhoeae* ~~at least twice~~ from another cause of STI, *H. ducreyi*. We describe the evolution of p*bla* since its emergence in *N. gonorrhoeae*, and its association with pConj. Gene loss and the appearance of novel TEM alleles influence the benefits and costs of p*bla* to the gonococcus. These traits are associated with the success and distribution of p*bla* variants within the gonococcal population.

~~*H. ducreyi* strains are divided into two clades/classes, which diverged ~1.9 million years ago (26); p*bla*.1- and p*bla*.2-like plasmids are found in Class I and Class II isolates, respectively. Thus, the independent acquisition of p*bla*.1 and p*bla*.2 by the gonococcus likely reflects separate events involving the two clades. Both interspecies transfers were accompanied by truncation of Tn2, suggesting the transposase is disadvantageous in the gonococcus.~~

In women, *N. gonorrhoeae* primarily causes cervicitis, while *H. ducreyi* causes ulcers at the vaginal entrance and cervix. In men, *N. gonorrhoeae* primarily causes urethritis and *H. ducreyi* mainly causes penile ulcers (43). Therefore, these species can occupy the same niche, providing ample opportunities for gene transfer. The presence of p*bla*.1 and p*bla*.2-like plasmids in *H. ducreyi* could indicate independent introductions of p*bla*.1 and p*bla*.2 into the gonococcus or independent emergence of p*bla*.1 in *H. ducreyi* and *N. gonorrhoeae*. Whilst we cannot reject either scenario, the independent emergence of p*bla*.1 in *H. ducreyi*

and *N. gonorrhoeae* through the deletion of *repB*/NEIS2964 allele 2 and *repB*/NEIS2964 allele 3, respectively, is the most parsimonious explanation.

~~Interestingly,~~ p*bla* is found relatively frequently in *N. gonorrhoeae* and *H. ducreyi*, which inhabit the genitourinary tract, but is seldom present in pathogens which inhabit the nasopharynx (*e.g. H. influenzae* and *N. meningitidis*); p*bla* was not detected in any non-invasive *Neisseria* spp. (41,158 isolates, 30 species). This could reflect the renal excretion of beta-lactams (44), favouring p*bla* carriage for bacteria inhabiting the urogenital tract compared with other sites. T~~Of note, t~~he meningococcal urethritis clade (NmUC) evolved from ST-11 *N. meningitidis* by acquiring genetic elements ~~(but not p*bla*)~~ from *N. gonorrhoeae* (45, 46). So far, p*bla* has not been reported in NmUC. H~~, h~~owever, we previously found an ST-11 *N. meningitidis* isolate harbouring ~~detected~~ p*bla* ~~in an~~ (1). This raises the possibility that NmUC might acquire p*bla*, which could provide an entry point of this plasmid into the meningococcal population, and the emergence of beta-lactamase producing *N. meningitidis* ~~that the plasmid could appear in NmUC~~.

Plasmids can be successful in bacterial populations either by ~~their ability to undergo~~ horizontal transmission and their ability to spread into diverse lineages, or through ~~their ability to undergo~~ vertical transmission ~~and~~ through their stable inheritance ~~be inherited stably~~ while being beneficial to their host. ~~We found that~~Our data indicates that the success of p*bla* depends both on its own mobility and its association with pConj variants that can effectively mediate its spread. Given the higher rates of pConj conjugation (>75%) compared with p*bla* mobilisation (~1%), the spread of p*bla* into a lineage is likely to be accompanied by pConj, maintaining the close association between these plasmids. Interesting, unlike many

other conjugative plasmids (47), pConj lacks entry exclusion, so p*bla* can enter bacteria already containing pConj, allowing proliferation of bacteria with successful p*bla*/pConj pairs, such as p*bla*.1/pConj.1, across the gonococcal population.

The most frequent and widespread p*bla* variant, p*bla*.1, is mobilised efficiently by common pConj variants and does not impose fitness costs. p*bla*.2 is also mobile but imposes fitness costs. Compared with p*bla*.1, p*bla*.2 has a second replication initiation protein and additional origins of replication (*i.e. ori2* and *ori3*) (1, 48) which might increase copy number and/or impose the fitness costs of p*bla*.2; plasmid Rep proteins can sequester host DNA replication machinery (41), causing fitness costs. We found that p*bla*.2 is present in lower prevalence in lineages than p*bla*.1, with evidence of a shift from p*bla*.2 to p*bla*.1 in a single lineage (Ng_cgc$_{400}$ 29) over time. Whilst the observed shift in p*bla* variants could reflect sampling bias, it is consistent with strains carrying p*bla*.2 being outcompeted by plasmid-free isolates or those with other p*bla* variants. Further evidence is provided by a longitudinal 10 year study of over 1,700 gonococcal isolates from a single city in China (Guangzhou), which reported a marked increase in p*bla*.1 (p*bla* Africa, from 18.4 to > 90% of isolates) with a concurrent fall in p*bla*.2 infections (from 81.6 to 7.6%) due to expansion of ~~a~~ successful clones harbouring p*bla*.1 (49).

p*bla*.3-associated lineages have undergone clonal expansion indicating its successful adaptation to the gonococcus. Phylogenetic analysis indicates that TEM-135 originally arose in p*bla*.2. However, despite increased resistance levels conferred by TEM-135, the fitness cost of p*bla*.2 has likely undermined the success of TEM-135 in this p*bla* variant. We found that p*bla*.3 evolved from TEM-135 carrying p*bla*.2 through gene loss. This prevented the

plasmid from being mobile, but with the trade-off of avoiding fitness costs. Consequently, the prevalence of p*bla*.3 in gonococci is not due to transfer between isolates, but through the expansion of p*bla*.3-carrying isolates, potentially through the increased beta-lactam resistance associated with TEM-135 and/or its association with an otherwise successful lineage. Further work is required to distinguish between these and other possibilities. ~~Consequently, p*bla*.3 has not spread in the gonococcal population but promoted the expansion of group of related lineages, likely though the increased beta-lactam resistance associated with TEM-135.~~

Since the emergence of gonococcal p*bla* in the 1970s, the evolutionary trajectory of this plasmid has been marked by its association with pConj variants that enable its spread through the population, the appearance of plasmid variants with minimal costs, and emergence of TEMs promoting higher resistance (*e.g.* TEM-135). A major concern is that the ESBL-permissive M182T substitution in TEM-135 is already widespread in gonococci (1, 8), especially in p*bla*.3.

The intimate relationship between p*bla* and pConj also highlights the threat posed by increased use of tetracyclines, as already witnessed in LMICs where gonococci have remarkably high plasmid carriage (7, 50). Similarly, the implementation of Doxy-PEP will increase selection for the carriage of pConj in gonococci and will thence select the isolates/lineages harbouring the plasmid. A significant threat is posed by isolates carrying pConj as well as chromosomal mutations conferring cefotaxime resistance (51). The spread of these isolates, as well as p*bla* (which itself could become an ESBL-plasmid) have the potential to undermine the successful treatment of cases and their contacts with third-

384 generation cephalosporins, the first-line antibiotics currently used to control ~~the spread of~~

385 gonococcal infection.

METHODS

Analysis of *Haemophilus* spp. and *Neisseria* spp. genomes

Tn*2*-carrying isolates of *Haemophilus* spp. and *Neisseria* spp. were identified querying Tn*2* (GenBank accession: LC091537.1) against *Haemophilus* (accessed 824/47/20254, 4,640 6,403 isolates, 12 species) and *Neisseria* (accessed 24/07/2024, 41,158 isolates, 33 species) sequences in PubMLST (23) (blastN word size: 11, scoring: reward: 2; penalty: -3; gap open: 5; gap extend: 2, sequence identity >99%, alignment length >50% query). p*bla*-like plasmids were confirmed by the presence of NEIS2960 (sequence identity >80%; alignment length >50%) and NEIS2358, and NEIS2961(1).

Plasmid variants

p*bla* and pConj were analysed in 15,52932 gonococcal WGS on PubMLST (accessed 28/07/2022, Supplementary Table 4)(1, 23) with isolates from 1928-2022 and 66 countries. NEIS2220 indicates the presence of pConj, and variants were defined according to gene presence/absence and specific alleles of plasmid genes (8). Due to the expansion of the genomic database, rather than using Ng_cp5s to define variants, we used a curated dataset of pConj sequences (8) together with pConj sequences in our dataset and clustered plasmids with Grapetree (52) to assign pConj variants. p*bla* variants were typed using the Ng_p*bla*ST scheme (1). For the population structure, isolates were grouped into core genome clusters according to the allelic profile of 1,668 core genes(40); isolates were grouped with a cut-off of 400 allelic differences (Ng_cgc_{400}).

Phylogenetic analyses

A subset of 414 p*bla*-carrying isolates conserving the ratio of p*bla* variants (70% p*bla*.1, 14% p*bla*.2, 16% p*bla*.3, Supplementary Table 32) (1) was selected to investigate the phylogenetic relationship of p*bla* variants. This included all p*bla*-containing isolates pre-dating 2000 (n=35). Isolates between 2000 and 2022 (n=379) were randomly selected using the r sample function (1). Snippy v4.6.0 mapped plasmid reads to *H. ducreyi* DMC64 p*bla* (minimum coverage, 4 and base quality, 25). Multiple sequence alignments were generated with snippy-core v4.6.0/snippy-clean v4.6.0. Maximum likelihood trees were generated using RaxML-ng v1.2.2 (53) with 100 bootstrap replicates, rooted at *H. ducreyi* DMC64 p*bla*, and visualised with ape (54) and ggtree (55, 56).

Structure predictions

Analysis of NEIS2962 and RSF1010 MobC (GenBank accession: S96966.1) homodimers and NEIS2962 with p*bla oriT* (38) were performed using AlphaFold 3 (57) and PyMol v2.5.4 (58). Charge distributions were visualised with the Adaptive Poisson-Boltzmann Solver (APBS) electrostatics tool (59).

Bacterial strains/growth

Strains and plasmids used in this study are listed in Supplementary Tables 54 and 65, respectively. *E. coli* DH5α was grown on Luria-Bertani (LB) agar or in liquid LB shaking at 180 rpm. *N. gonorrhoeae* was grown on Gonococcal Base Media (GCB) agar plates or liquid media (GCBL) (60) supplemented with 1% Vitox (Oxoid) at 37°C in 5% CO_2. *H. ducreyi* was grown on chocolate agar plates supplemented with 1% IsoVitaleX at 35°C in 5% CO_2. Antibiotics were added as follows: for *E. coli*, carbenicillin 100 μg/ml; for *N. gonorrhoeae*,

carbenicillin 2.5 μg/ml; erythromycin 1 μg/ml; kanamycin 50 μg/ml, and tetracycline 2 μg/ml.

Characterisation of *H. ducreyi* plasmids

Genomic DNA was isolated from *H. ducreyi* by harvesting bacteria from plates and the DNeasy Blood/Tissue Kit (Qiagen) with the modifications that cells were incubated in lysis buffer with 20 mg/ml lysozyme at 37°C for 2 hours and then proteinase K overnight at 56°C. Plasmids were analysed by Sanger sequencing using primers listed in Supplementary Table 7~~6~~. Sequence similarity of plasmid sequences was assessed with ClustalW with default parameters (61) and sequences were mapped onto *H. ducreyi* DMC64 p*bla* using Snapgene v6.1.1 (Insightful Science; available from snapgene.com) to investigate deletion sites.

Transformation of gonococci

For electroporation, bacteria grown on GCB agar were resuspended in PBS (Sigma), adjusted to $5x10^7$ CFU/ml then washed three times with 20% glycerol / 1% MOPS (Sigma); electroporation was performed with 2.5 kV, 200 Ω, 25 mF. Cells were recovered in 1 ml of GCBL with 2% Vitox and plated on GCB agar. Plates were incubated for 3 hours, cells collected, and then transferred to selective media.

Δ*pilD*::*ermC* and Δ*pilD*::*aph* constructs were transformed into *N. gonorrhoeae* as described previously (29, 31). In brief, 1 μg of DNA was spotted onto plates, allowed to dry, and bacteria streaked over the spots. Plates were incubated for 8 hours, then bacteria were transferred onto selective agar. Transformants confirmed by PCR/Sanger sequencing.

Plasmid modifications

All primers are listed in Supplementary Table ~~8~~7. To generate p*bla*.1^{iso} and p*bla*.3^{iso}, p*bla*.2 was cut with *Hin*dIII-HF and *Pvu*II-HF (NEB), respectively. p*bla*.1^{iso} was amplified with primers TE18/19 and PrimeSTAR GXL polymerase (Takara Bio); Gibson assembly was performed with primers TE20/21. p*bla*.3^{iso} was amplified in two fragments with TE7/TE17 and TE9/TE16. Plasmids were assembled using Gibson Hifi (NEB) and transformed into *E. coli* DH5α.

Point mutations in *bla*TEM were introduced using the RAIR method (62). PCRs with primers (TE56/TE57, p*bla*.2 TEM-135; TE63/TE64, p*bla*.2$^{TEM\text{-}1\ P14S}$; TE65/TE66, p*bla*.3$^{TEM\text{-}1}$) were performed using Herculase II polymerase (Agilent), with p*bla*.2 or p*bla*.3 as template. Products were purified (Promega Wizard PCR Clean-up) and transformed into *E. coli* DH5α.

tetM^{+} pConj.7 was constructed by amplifying *tetM* from *N. gonorrhoeae* WHO N using primers TE34/TE35. Flanking regions were amplified with primers TE36/37 and TE38/39, then joined by Gibson assembly; the product was amplified with TE36/39, then introduced into *N. gonorrhoeae* NG028 by transformation. All constructs were confirmed by sequencing.

Conjugation and mobilisation assays

Donor (Δ*pilD*::*ermC*) and recipient (Δ*pilD*::*aph*) strains grown overnight were inoculated in 5 ml GCBL/1% Vitox at an OD_{600} of 0.1 and grown to mid-exponential phase (OD_{600} 0.6 - 0.8). The bacterial density was adjusted to 10^8 CFU/ml and donor and recipient strains mixed in a 10:1 ratio. Bacteria (5 µl) were spotted onto GCB agar and incubated for 6 hours at 37°C, 5% CO_2, harvested in 200 µl GCBL, then plated to GCB agar with antibiotics. Conjugation and mobilisation frequencies were defined as the number of transconjugants/recipients (n=3).

Plasmids used in the different assays are listed in Supplementary Table ~~6~~5 together with the WGS of the corresponding isolates available on PubMLST.

Competition assays to determine fitness costs

Plasmids were introduced into FA1090 Δ*pilD*::*ermC* and competed against FA1090 Δ*pilD*::*aph* (n=4). Bacteria in PBS were adjusted to an OD_{600} 1, mixed 1:1, diluted to 10^5 CFU/ml and added to 200 µl Fastidious Broth (63) then grown at 37°C, 5% CO_2, shaking at 180 rpm. After 24 hours, strains were enumerated by spotting on selective media. Fitness costs (w) were calculated by:

$$w = \frac{ln(N_f/N_i)}{ln(N_{f,pbla}/N_{i,pbla})}$$

(w, relative fitness of p*bla*$^+$ *vs*. p*bla*$^-$ strains; $N_i$and N_f, p*bla*$^-$ strain at the beginning/end, respectively; $N_{i,pbla}$and $N_{f,pbla}$, same for p*bla*$^+$ strain).

Antibiotic susceptibility testing

Penicillin G MICs were assessed using the broth microdilution method(64) in 96-well plates with 2-fold Penicillin dilutions in water (50 µl); strains grown overnight on GCB agar were resuspended in PBS (Sigma), then diluted in 2x FB/2% Vitox to 10^5 CFU/ml. Bacteria (50 µl) were transferred into each well and incubated for 24 hours.

SDS page and Western blot analysis

Bacteria were grown to mid-exponential phase, added to an equal volume of 2x SDS-PAGE buffer, run on 12% SDS-polyacrylamide gels, and transferred to Protan nitrocellulose membranes (GE Healthcare) using the Trans-Blot Turbo System (Bio-Rad). Membranes were blocked in PBS/0.5% Tween-20/5% milk, washed thrice and incubated with the primary antibodies (Rabbit anti-RecA, Abcam, ab63797, 1:5,000; Mouse anti-TEM, Abcam, 8A5.A10, 1:1,000) for 2 hours. After washing, membranes were incubated with secondary antibodies (LI-COR Biosciences, 925-68071 IRDye® 680RD Goat anti-Rabbit IgG and 925-32210 IRDye® 800CW Goat anti-Mouse IgG) at a final dilution of 1:10,000 for 1 hour, washed, then imaged using LI-COR Biosciences.

Plasmid copy number

Copy number of *recA* and plasmid *tnpR* were quantified using the QX200 Droplet Digital PCR system (ddPCR, Bio-Rad) as described previously(65). ddPCR contained 1x EvaGreen super mix (Bio-Rad), and TE79/TE80 (*recA*, Supplementary Table 8~~7~~) or TE81/TE82 (*tnpR*, Supplementary Table 8~~7~~). After thermal cycling, data were analysed using the QX200 Droplet Reader with QuantaSoft software (Bio-Rad).

TEM mRNA expression

p*bla*.1$^{TEM\text{-}1}$ carrying isolates were sub-cultured to mid-exponential phase in 5 ml GCBL/1% Vitox. Cells were harvested from 1 ml of culture and RNA was extracted using the Qiagen RNeasy Mini Kit together with the RNA protect Reagent (Qiagen, #76506) and on column RNase-free DNase I (Qiagen, #79254) treatment according to the manufacturer's instruction (protocol RY28). RNA was subsequently reverse transcribed to cDNA using the LunaScript RT

SuperMix Kit (NEB, #E3010). The cDNA was used in ddPCRs with primers TE79/80 (*recA*) and TE97/98 (*bla*TEM, Supplementary Table 8~~7~~). The no-RT and no template reactions served as negative controls.

Statistics and data analysis

Data analysis was performed in R version 4.1.1 using base R and the tidyverse package(66). Plots were generated with ggplot2 (67). A p value <0.05 was considered statistically significant.

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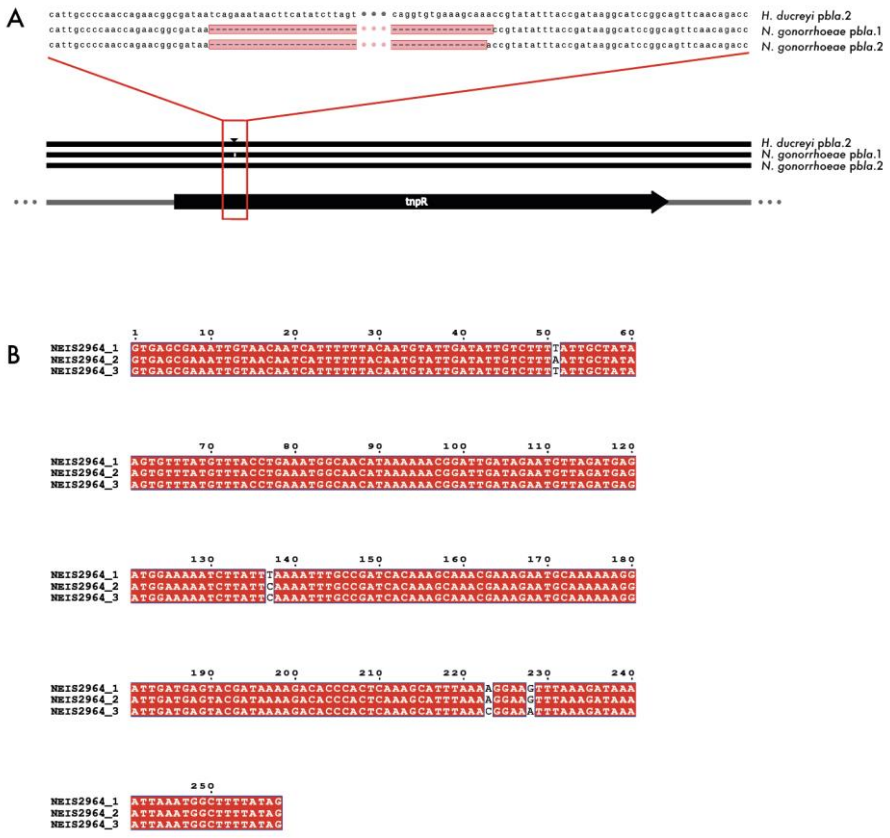
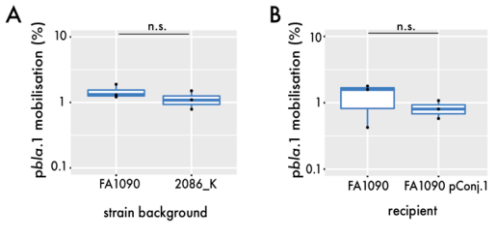
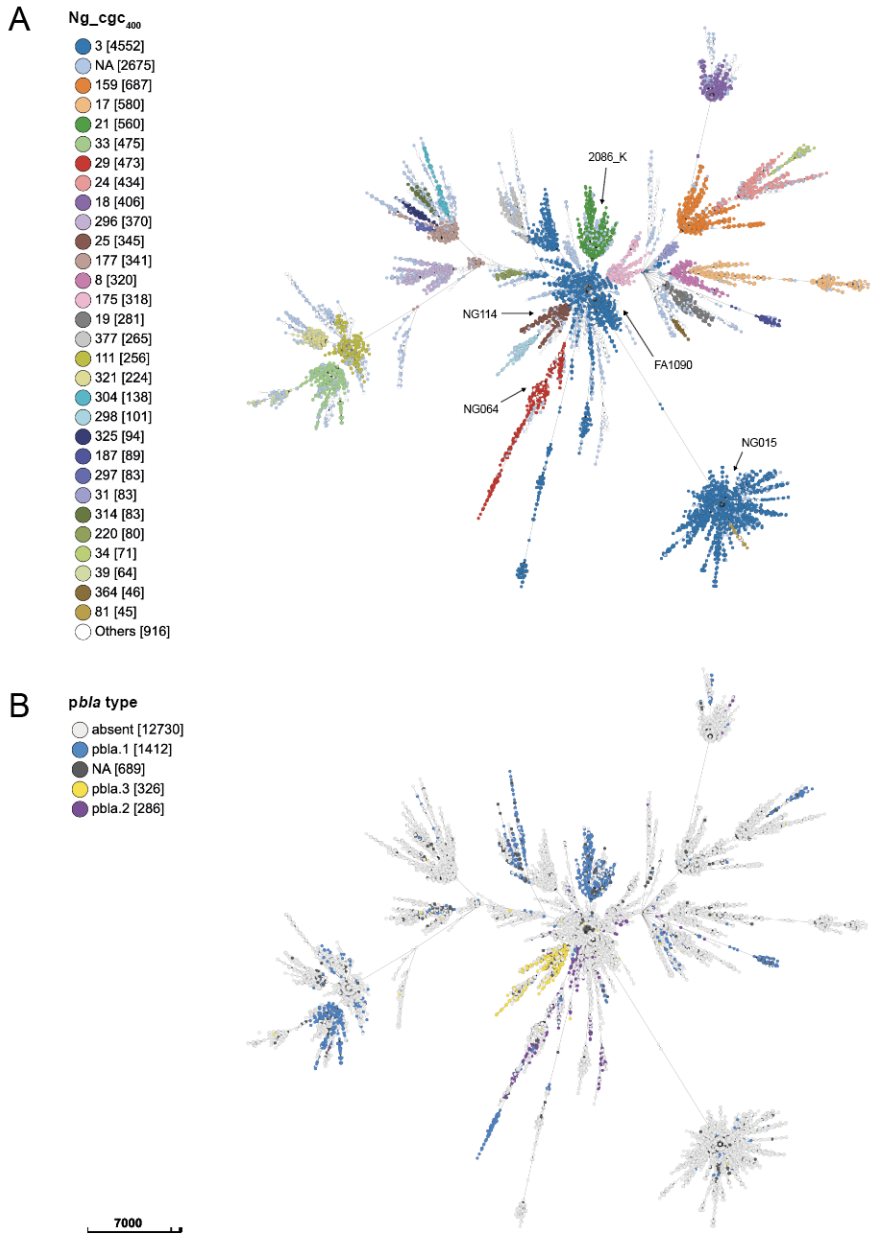
SUPPLEMENTARY FIGURES



Supplementary Figure 1: (A) Analysis of the Tn*2* deletion region: the truncation affecting *tnpR* in gonococcal p*bla*.1 and p*bla*.2 differs by a single nucleotide. Schematic representation of *tnpR* sequences of gonococcal and *H. ducreyi* p*bla*s is shown on the bottom with the SNP displayed as a white line and the site of the Tn*2* truncation in relation to *H. ducreyi* p*bla* indicated with a triangle. Displayed above is the nucleotide alignment of gonococcal p*bla* to *H. ducreyi* DMC64 p*bla*.2. (B) Sequence alignment of NEIS2964 alleles -1 to -3) with SNPs highlighted in white.



Supplementary Figure 2: p*bla*.1 mobilisation by pConj.1 in isogenic matings. (A) p*bla*.1 mobilisation in the *N. gonorrhoeae* strains FA1090 and 2086_K (Welch two-sample t-test, p = 0.66). (B) Mobilisation rates of p*bla*.1 into FA1090 with or without pConj (Welch two-sample t-test, p = 0.87).



Supplementary Figure 3: Minimum spanning trees of *N. gonorrhoeae* clustered by core genome allelic differences with distribution of p*bla* variants. Individual dots represent isolates that are coloured according to Ng_cgc_{400} (A) or p*bla* variant carried (B). Strains used in this study are indicated in panel A.

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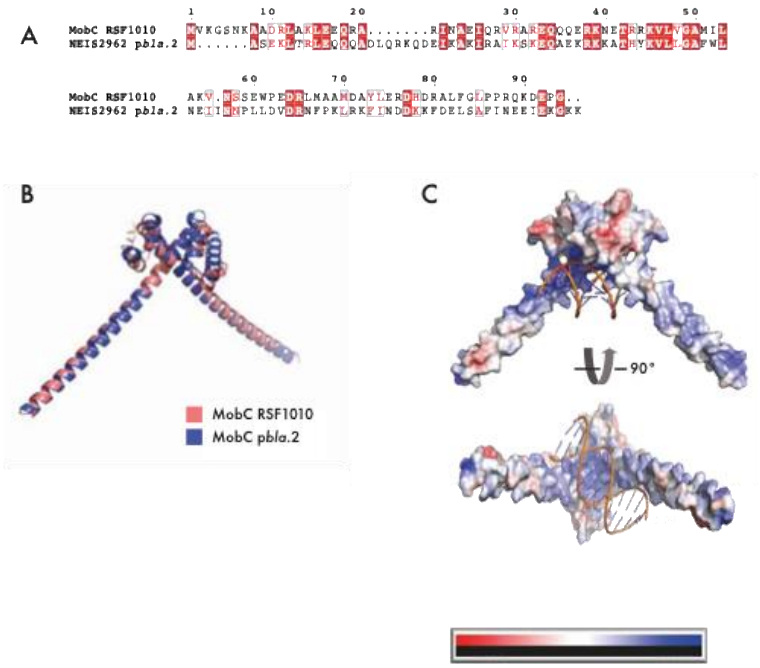

Supplementary Figure 4: (A) Alignment of MobC from *E. coli* plasmid RSF1010 (Genbank accession: S96966.1) and NEIS2962 from p*bla*.2 (Genbank accession: NZ_LT591911). Amino acid sequences were aligned with COBALT(2) and the alignment visualised with Esprit (3). Identical residues are shown in white on red background, residues with a similarity score >0.7 are framed in blue and the remaining residues are shown in black. (B) Superimposed AlphaFold structure prediction of MobC from the *E. coli* plasmid RSF1010 (salmon, Genbank accession: S96966.1) and NEIS2962 (blue) dimers (Match Align: 677.7, RMSD: 0.775Å) (C) Electrostatics prediction of NEIS2962 homodimer with *oriT* sequence using the Adaptive Poisson Boltzman Solver Electrostatics Plugin. Negatively and positively charged regions are shown in red and blue, respectively.

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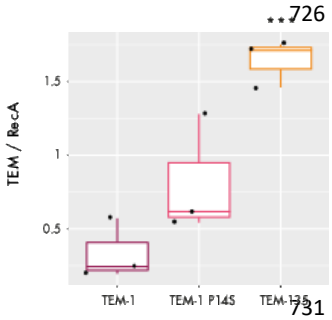

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Supplementary Figure 5: Cellular levels of different TEM variants in an isogenic FA1090 background. TEM/RecA ratios of whole cell lysates were visualised by Western blot analysis and quantified with the LI-COR system (one-way ANOVA with Tukey multiple comparisons, n.s. p>0.05; *** p<0.001).

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