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A low affinity penicillin-binding protein 2x is required for heteroresistance in *Streptococcus pneumoniae*

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Abstract

Heteroresistance to penicillin in *Streptococcus pneumoniae* is the ability of subpopulations to grow at a higher antibiotic concentration than expected from the minimal inhibitory concentration (MIC). This may render conventional resistance testing unreliable and lead to therapeutic failure. We investigated the role of the primary β-lactam resistance determinants, penicillin binding proteins PBP2b and PBP2x and secondary resistance determinant PBP1a in heteroresistance to penicillin. Transformants containing PBP genes from heteroresistant strain Spain$^{23F}_{2349}$ in non-heteroresistant strain R6 background were tested for heteroresistance by population analysis profiling (PAP). We found that *pbp2x,* but not *pbp2b* or *pbp1a* alone, conferred heteroresistance to R6. However, a change of *pbp2x* expression is not observed and therefore expression does not correlate with an increased proportion of resistant subpopulations. Additional *ciaR* disruption mutants which have been described to mediate PBP-independent *β*-lactam resistance revealed no heteroresistant phenotype by PAP.

We also showed, that the highly resistant subpopulations (HOM*) of transformants containing low affinity *pbp2x* undergo an increase in resistance upon selection on penicillin plates which partially reverts after passaging on selection-free medium. Shotgun proteomic analysis showed an upregulation of phosphate ABC transporter subunit proteins *pstS*, *phoU*, *pstB* and *pstC* in these highly resistant subpopulations.

In conclusion, the presence of low affinity pbp2x enables certain pneumococcal colonies to survive in the presence of beta lactams. Upregulation of phosphate ABC transporter genes may represent a reversible adaption to antibiotic stress.

Introduction

Streptococcus pneumoniae is an important human pathogen causing up to 11% of child deaths per year (1). Although initially very susceptible to penicillin, resistance to this antibiotic in *S. pneumoniae* has become a global concern within a few decades. Today, a small number of resistant clones dominate the global resistance epidemiology (2, 3). The three main penicillin resistance determinants are altered penicillin-binding proteins PBP2x, PBP2b and PBP1a, which are responsible for the final crosslinking of the peptidoglycan in the bacterial cell wall (4, 5). PBP variants with low affinity to penicillin are acquired by horizontal gene transfer followed by homologous recombination events with commensal streptococci as donor species, giving rise to mosaic genes (4, 6-8). As detected for a clone of serotype 23F, which spread intercontinentally, the presence of low affinity variants confers increased penicillin resistance (9).

Besides PBPs, mosaic structures in the first cell wall branching enzyme (MurM) allow pneumococci to synthesize branched cell wall muropeptides which contribute to high penicillin resistance in some strains (10, 11). In resistant laboratory mutants, mutations in the two-component signal transduction system CiaRH (Competence induction and altered cefotaxime susceptibility) have been identified that also mediate β-lactam resistance. In addition, CiaRH is implicated in maintenance of cell integrity, competence and virulence (12-14). Thus many resistance components have to be optimized for high penicillin resistance to occur. However, other resistance determinants have been occasionally described in resistant strains, too (15).

Heteroresistance is thought to facilitate the development of high penicillin resistance (16). A heteroresistant bacterial strain has one or several subpopulations at a frequency of 10^{-7} to 10^{-3} which can grow at higher antibiotic concentrations than predicted by the minimal inhibitory concentration (MIC) for the majority of cells, which

all are identical genetically. Most studies focus on heteroresistance to methicillin, oxacillin and vancomycin in staphylococci (17-23) but the phenomenon has been described for pathogens of various species such as *Acinetobacter baumanii* (24, 25), *Pseudomonas aeruginosa (26, 27)*, *Enterococcus faecium* and *Mycobacterium tuberculosis (28, 29)*, but also fungi such as *Cryptococcus* spp (30, 31). In pneumococci, heteroresistance has been reported to penicillin and fosfomycin, and is likely to be produced via distinct mechanisms (16, 32). Understanding heterogeneity between single cells is challenging as conventional assays of microbial populations consider averaged values of thousands or millions of cells (33). Therefore, the mechanism of heteroresistance remains unclear to date. A categorization into four classes according to the frequencies of subpopulations with higher resistance has been suggested based on work on methicillin resistant staphylococci (23). For pneumococci, class II and class III heteroresistance have been observed (16). A strain with class II heteroresistance grows subpopulations with higher resistance at a frequency of 10^{-6} to 10^{-4}. Furthermore, presence of several subpopulations with different MICs is characteristic leading to a continuous decreased frequency of subpopulations in the PAP curve. In contrast, in a class III heteroresistant strain one subpopulation, represented by a plateau in the PAP curve, is predominant.

In this study we uncover a mechanism of penicillin heteroresistance in *S. pneumoniae* by transferring *pbp* genes between heteroresistant and non-heteroresistant strains and by a shotgun proteomic approach to study the highly resistant subpopulations.

Materials and Methods

Bacterial strains and growth conditions. Spain$^{23F}_{2349}$ and Spain^{6B}-two international reference strains of *Streptococcus pneumoniae*, the laboratory strain R6 and a selection of transformants and progeny of these strains were used in this study. All strains used to investigate the heteroresistance phenomenon are listed in Table 1. Strains used for cloning are listed in Table S1. Bacterial conservation and growth procedures have been described before and are briefly mentioned in Materials and Methods in the supplemental material (16).

Antibiotic Susceptibility Testing. MICs were determined by E-test method (bioMérieux, Switzerland) according to the manufacturer's protocol. All isolates were tested in triplicate and the plates were incubated at 5% CO_2.

DNA techniques. Pneumococcal chromosomal DNA or cell pellet were used as PCR templates (34). DNA fragments were amplified with high-fidelity iProof polymerase (BioRad). Constructs were fused with either iProof or Phusion high-fidelity polymerase (Thermo Scientific) as described elsewhere (35). Introduced genes were sequenced as described here (36) to confirm correct insertion and absence of additional mutations. DNA oligonucleotides used for PCR and sequencing are listed in Table S2.

Transformation procedure. Transformation of *S. pneumoniae* was performed according to published procedures (37, 38). The β-lactam concentrations used to select mosaic *pbp2b* and *pbp2x* are specified below. Streptomycin (CAS 3810-74-0), kanamycin (CAS 25389-94-0) and spectomycin (CAS 22189-32-8) all from Sigma were used at 200 µg/ml.

Introduction of low-affinity mosaic PBP2b$_{2349}$. First, a 1858 bp gene fragment containing the mosaic block was amplified from *S. pneumoniae* Spain$^{23F}_{2349}$ using Taq polymerase (Qiagen) and primers pbp2b_for and pbp2b_rev. Then, the PCR

product was cloned into pGEM-T Easy (Promega) creating plasmid pGEM-2bRes. *E. coli* DH5α was transformed with the ligation product and selected with X-gal/IPTG-LB agar plate containing 100 µg/ml ampicillin. Next, *S. pneumoniae* R6 was transformed with pGEM-2bRes, the resistant clones were selected on CSBA plates containing 0.05 µg/ml piperacillin (CAS 59703-84-3). One transformant, R6::*pbp2b*$_{2349}$, which contains a complete mosaic block (codon 982 to 1472) from Spain$^{23F}{}_{2349}$ was used for further study.

Introduction of mosaic PBP1a$_{2349}$. Transformants R6::*pbp1a*$_{2349}$ and R6::*pbp2b1a*$_{2349}$ were constructed as described before (34). Janus cassette was amplified from R6*pbp2x*$_{T338G}$*pbp1a*::Janus with Ja-*pbp1a*_for and Ja-*pbp1a*_rev. Presence of *pbp1a*$_{2349}$ was verified in resulting mutants by DNA sequencing. Construction of R6::*pbp2b2x*$_{2349,}$ R6::*pbp2b2x1a*$_{2349}$, mosaic PBP2x$_{6B}$ and PBP2x$_{2349}$ transformants and of loss-of-function CiaR derivative was done as described in Materials and Methods in the supplemental material.

Population analysis profiles (PAP). PAP were performed for penicillin as described earlier (16). Briefly, strains were streaked out from frozen stock on CSBA plates and incubated for 24 h in a 5% CO_2 atmosphere at 37°C. Then an overnight culture of 5 ml brain heart infusion (BHI) (BD Difco) with 5 % FBS (Biochrom AG, Germany) was prepared and inoculated with 5 – 20 colonies. 100 µl of overnight culture was subcultured in 5 ml BHI + FBS and grown to mid log phase (OD_{600nm} = 0.7). The culture was diluted 10^{-2} to 10^{-4} and 10^{-6} in PBS (pH = 7.4) and 100 µl plated on Müller-Hinton broth (MHB) agar plates (BD Difco) with 5% sheep blood. Penicillin G (CAS 113-98-4) concentrations in MHBA plates ranged from 0 – 5.0 µg/ml. Colonies were counted by eye after 48 h of incubation at 37 °C in 5% CO_2.

Growth curves. Growth curves from double-mutants R6::*pbp2b2x*$_{2349}$ and R6::*pbp2x1a*$_{2349}$ and their HOM* progeny strains were obtained in BHI+FBS 5% as

155 described before (39). A total of 5 x 10^5 CFU from frozen stock was used for
156 inoculation.

157 **Gene expression studies.** Bacteria were grown overnight in BHI+FBS 5% for 9h at
158 37°C. Attention was paid that OD_{600nm} of overnight culture did not exceed 0.8. 100 µl
159 of the overnight culture was rediluted in 5ml BHI+FBS and grown to an OD_{600nm} =
160 0.5. 5ml of the culture were added to 10ml RNA protect (Qiagen), RNA extracted and
161 expression of *pstS*, *pstB*, *pbp2x* and *pbp2b* was quantified as described elsewhere
162 by real-time RT-PCR (39). For primers and probes see Table S2. The remaining
163 culture was pelleted, then resuspended in 200 µl PBS + 15% glycerol and frozen at –
164 80°C.

165 **Detection of penicillin-binding proteins and comparison of protein expression**
166 **patterns by LC-MS/MS.**

167 Penicillin-binding proteins were detected as described previously (38). A brief
168 description is given in Materials and Methods in the supplemental material. The LC-
169 MS/MS method is described in the supplemental material, too.

170

Results

Heteroresistance to penicillin depends on the presence of a low-affinity PBP2x. First, we looked at the role of the primary and secondary resistance determinants, PBP2b, PBP2x and PBP1a variants with low affinity to penicillin, in heteroresistance to penicillin. Transformants carrying mosaic blocks of the *pbp* genes of the heteroresistant strain Spain$^{23F}_{2349}$ in the background of the non-heteroresistant laboratory strain R6 were characterized in population analysis profiles (PAP). Comparing the single-transformants carrying *pbp2b*$_{2349}$ or *pbp1a*$_{2349}$ no difference in the heteroresistance phenotype from that of strain R6 was observed (Figure 1 A). However, R6::*pbp2x*$_{2349}$ showed class III heteroresistance, characterized by growth of one subpopulation with higher resistance at a frequency of 10^{-4} to 10^{-3} (Table 1; Figure 1A). The double-transformants carrying *pbp2b*$_{2349}$ and *pbp1a*$_{2349}$ in R6 background showed no heteroresistance, meaning no subpopulations growing with higher resistance than the MIC (Figure 1 B). However, the combination of *pbp2b*$_{2349}$ and *pbp2x*$_{2349}$ led to class II heteroresistance, characterized by presence of several subpopulations at a frequency of 10^{-4} to 10^{-1} (Table 1). The transformant with *pbp2x*$_{2349}$ and *pbp1a*$_{2349}$ showed higher resistant subpopulations at a frequency 10^{-5} to 10^{-4}. Uniting the three resistance genes in a triple-transformant led to a phenotype close to wild-type Spain$^{23F}_{2349}$ with a class II heteroresistance pattern (Figure 1 C). Replicates of PAP of R6 wild-type, *pbp* single, double and triple mutants were highly reproducible (Figures S1 and S2). Thus, all transformants containing *pbp2x*$_{2349}$ independent on the presence of other PBP genes from *S. pneumoniae* 2349 showed heteroresistance phenotypes, but those with other PBP genes did not.

Heteroresistance is also conferred by *pbp2x* of Spain^{6B}-2. To assess whether different C-terminal regions in *pbp2x* affect the heteroresistance phenotype, we additionally created double-transformants carrying *pbp2x*$_{6B}$ from non-heteroresistant

strain Spain^{6B}-2 instead of *pbp2x*$_{2349}$ from heteroresistant strain Spain$^{23F}_{2349.}$ The pbp2x sequence differences between both strains are shown in Figure S3. Performing PAP, we found that combining *pbp2b*$_{2349}$ and *pbp2x*$_{6B}$ in R6 background (Figures 2A and S4) leads to class II heteroresistance as observed for the combination *pbp2b*$_{2349}$ plus *pbp2x*$_{2349}$ (Figure 1 B). Identical findings were also obtained when combining *pbp1a*$_{2349}$ and *pbp2x*$_{6B}$ as this led to class III heteroresistance as seen with *pbp2b*$_{2349}$ plus *pbp2x*$_{2349}$ (Figures 2B and S4). Introduction of *pbp2x*$_{6B}$ into R6 therefore led to the identical heteroresistance pattern as did *pbp2x*$_{2349.}$ Compared to Spain^{6B}-2 and Spain$^{23F}_{2349,}$ non-heteroresistant R6 has an unique transpeptidase domain while its PASTA domain is nearly equal to Spain^{6B}-2 (with the exception of Amino acid No. 693; Figure S3). This therefore shows that the transpeptidase domain of PBP2x but not the two C-terminal PASTA domains are involved in the heteroresistance phenotype.

No influence of *ciaR* disruption on heteroresistant phenotype. As the CiaRH system can mediate PBP-independent *β*-lactam resistance we investigated whether heteroresistance was lost upon silencing the CiaRH system by disruption of the response regulator gene *ciaR*. It was found that *ciaR* disruption did not affect MICs (Table 1) although in PAP R6Δ*ciaR* displayed a slightly higher susceptibility to penicillin than R6 WT. However, R6*pbp2x*$_{2349}$Δ*ciaR* retained heteroresistance to penicillin as compared to R6::*pbp2x*$_{2349}$ (Figures 2C and S4).

PAP of highly resistant sub-populations (HOM*) progeny strains show shift towards higher resistance which reverts partially after passaging on selection-free media. From PAP, highly resistant sub-populations (HOM*) progeny strains were obtained. HOM* progeny strains were grown from a single colony picked from a PAP plate with highest or second highest penicillin concentration showing growth. The colony picked from PAP of the original transformant was HOM*1, progeny

selected from its PAP, HOM*2. The third generation HOM*3 was subjected to PAP and also to 15 passages on antibiotic free medium to obtain HOM*3p. For R6::*pbp2b2x*$_{2349}$ HOM*1 a shift towards higher penicillin resistant subpopulations was observed (Figure 3 A). The MIC determined for the majority of the population was 0.094 µg/ml penicillin. For HOM*2 progeny too, a shift towards higher resistance to penicillin was observed for the subpopulations without change in the MIC. A subsequent clone, HOM*3, however, possessed subpopulations with similar resistance to penicillin to HOM*2 and also the MIC of HOM*3 progeny was the same as for HOM*2 (Figure 3 A). After 15 passages on selection-free medium the MIC determined for R6::*pbp2b2x*$_{2349}$ HOM*3p had reverted to the initial level of 0.064 µg/ml penicillin (Table 1). Also, increase of resistance in subpopulations and MIC for the entire population was observed for HOM* progeny of R6::*pbp2x1a*$_{2349}$ (Figure 3 B). Again, no further increase in resistance was obtained for HOM*3 compared to HOM*2. Also MICs as determined for the majority of cells increased from 0.047 µg/ml penicillin for HOM*1 to 0.38 µg/ml penicillin for HOM*3 and reverted to 0.064 µg/ml for HOM*3p. For HOM*2, HOM*3 and HOM*3p subpopulations grew at concentrations up to 2 to 7 times MIC determined for the majority of cells within the inhibition zone of E-Test (Table 1). Again, replicates of PAP of R6::*pbp2b2x*$_{2349}$ and its HOM were highly reproducible (Figures S5). Therefore, both R6::*pbp2b2x*$_{2349}$ (Figure 3A) and R6::*pbp2x1a*$_{2349}$ (Figure 3B) shift towards higher resistance despite the heteroresistance class differ among the two original transformants (Figure 1B). Also, MIC values measured for HOM*3p switch back to initial levels (Table 1).

Altered growth for HOM* progeny strains

For PAP, CFU are counted after 48 h of incubation at 37°C to account for potential reduced growth of the subpopulation strains with higher resistance. However, to characterize more precisely differences in growth phenotypes for double-

transformants, HOM*3 and HOM*3p progeny growth curves of these strains were obtained. The original strains both grew to a maximum OD_{450nm} of 0.3 (Figure S6). However, the R6::*pbp2b2x*$_{2349}$ HOM*3 strain showed a tendency to grow to a higher OD than the original transformant (Figure S6 A), whereas for R6::*pbp2x1a*$_{2349}$ the opposite was observed. The R6::*pbp2b2x*$_{2349}$ HOM*3p strain grew in a similar way to the original transformant. Finally, HOM*3p progeny R6::*pbp2x1a*$_{2349}$ exhibited clearly impaired growth (Figure S6 B).

Altered protein expression levels in highly resistant sub-population progeny strains.

In order to identify components that might be responsible for the highly resistant subpopulations, the protein profiles of R6::*pbp2b2x*$_{2349}$ original transformant and its HOM*3 progeny were investigated by a shotgun liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. All of the quantifiable proteins (899 in total) had expression differences smaller than 1.5 (0.5 in LOG2 values as displayed in Figure 4A). A small fraction of fifteen proteins had a statistically apparent expression difference between the two samples, $p<0.05$ (Students T-Test) (Supplementary table S3). Within these, four had a ratio (HOM/WT) of >0.25 and were annotated as *pstS*, *phoU*, *pstB* and *pstC*, which are all subunit proteins of the same phosphate ABC transporter operon (Figure 4A). No significant differences were observed for the penicillin-binding proteins in this shotgun proteomic analysis. Expression of two of the four genes (*pstS* and *pstB)* was subsequently quantified by real time RT-PCR for strain R6::*pbp2b2x*$_{2349}$ and its HOM*1 and HOM*3 progeny (Figure 4 B). Whilst no difference in gene expression was measured for HOM*1 progeny, HOM*3 progeny expressed *pstS* and *pstB* to about 100 fold higher levels compared to the unselected population. We were able to confirm the increased expression for *pstS* and *pstB* for the wt (low expression) and HOM*3 (high

expression) in two additional lineages (Figure S7). As for HOM*1, the findings are in contrast as compared to the original experiment (Figure 4B) as we observed an increased expression. However, heterogeneity for HOM*1 may be interpreted as a 'transition state' between wt and HOM*3. RT-PCR results therefore matched the LC-MS/MS results showing significant upregulation of *pstS* and *pstB* in HOM*3. No difference in *pstS* and *pstB* expression was detected in R6 WT compared to the single and double mutants (data not shown).

No differences in the expression of penicillin-binding proteins

Although LC-MS/MS did not indicate any differences in the expression of the penicillin-binding proteins, their expression was quantified and compared between the double-transformants and their HOM*3 progeny strains using a different method. Production of PBPs was determined by staining with BocillinFL, a fluorescence-labeled β-lactam, and separation by SDS-PAGE. However, no increase in PBP was detected in HOM*3 progeny compared to the original double transformants (Figures 4 C). This was also true when measuring *pbp* gene expression by real time RT-PCR (Figure 4D).

Discussion

Heteroresistance describes the presence of one or several subpopulations of bacterial cells within a clonal strain that can grow at higher antibiotic levels than determined for the majority of cells. The phenomenon has been described for pneumococci without shedding light on the molecular mechanism (16). In this study we aimed to identify the relevant mechanisms to produce heteroresistance to penicillin.

Our data suggest that a low-affinity variant of PBP2x is required for a heteroresistant phenotype, which therefore assumes a key role in heteroresistance to penicillin. This finding is similar to previous work in staphylococci where heteroresistance to methicillin was observed upon insertion of *mecA* encoded PBP2a (19, 22). Interestingly we found the combination of low-affinity PBPs to determine the heteroresistance class of a strain and therefore the frequency of heteroresistant subpopulations as established in *S. aureus* (23). As observed previously the expressed heteroresistance class is a stable phenotypic trait (16, 23). PBP2b and PBP2x are monofunctional enzymes catalyzing transpeptidation only, whereas PBP1a exhibits transpeptidation and transglycosylation activity. It has been suggested that at penicillin concentrations close to the MIC, transglycosylation activity of PBP1a, not targeted by β-lactams, confers a critical degree of cell wall integrity for growth as peptidoglycan is incompletely cross-linked as PBP transpeptidase activity is hampered by penicillin (40, 41). Hence, in R6::*pbp2b2x*$_{2349}$ transformant, only the transpeptidase activity of the susceptible PBP1a$_{R6}$ is inhibited by penicillin. The low-affinity PBP2x$_{2349}$ might to some degree replace this function (42). In R6::*pbp2x1a*$_{2349}$ however, the essential PBP2b is targeted by penicillin (43). The subpopulations with higher resistance arise therefore with lower frequency, but when they occur they can grow to higher resistance levels. This is in agreement with

319 the observation that modified PBP1a are required for high penicillin resistance (44).
320 We report low-affinity PBP2x to be an essential tool in the production of penicillin
321 heteroresistant phenotype.
322 It has been hypothesized before, that auxiliary resistance genes in concert with low-
323 affinity PBP-variants produce a heteroresistant phenotype (16). Therefore we also
324 disrupted *ciaR* to test the influence of silenced CiaRH system, which mediates PBP
325 independent β-lactam resistance (12), on the heteroresistant phenotype. We found
326 heteroresistance to be conserved and conclude that the CiaRH system has a
327 negligible effect on the phenomenon. A previous study reports that total PBP
328 amounts found within bacteria do not differ between resistant and susceptible
329 pneumococci, nor does the amount increase when a subinhibitory concentration of
330 penicillin is present in the growth medium (45). We confirm this finding, as we could
331 not detect increased amounts of PBP or overexpressed *pbp* genes.
332 However, proteomic analysis of R6::*pbp2b2x*$_{2349}$ original transformant and its HOM*3
333 progeny revealed a significant overexpression of some phosphate ABC transporter
334 subunit proteins. The function of most of these components is unknown. However,
335 increased expression of *pstS* has also been identified by proteomic analysis for a
336 clinical isolate of serotype 23F, recently (46). This is intriguing as the common finding
337 between our group and that of Soualhine *et al*, clearly shows the importance of *pstS*
338 for both penicillin resistance and heteroresistance within *S. pneumoniae*. Soualhine
339 *et al* furthermore described an excellent correlation between resistance and
340 increased expression of *pstS* by RT-PCR (46). In contrast, we did not find any
341 expression differences in R6 WT compared to the single and double mutants.
342 However, if and how the presence of different penicillin binding proteins affects *pstS*
343 expression has to be further investigated in the future.

In conclusion, we show the importance of classical resistance mechanisms, represented by a low-affinity variant of PBP2x in the phenomenon of heteroresistance to penicillin. Furthermore, we detected increased expression of phosphate ABC transporter genes in the HOM* strains representing a reversible adjustment to antibiotic stress. Improved understanding of the mechanism of heteroresistance may lead to an improved diagnostics and to an adjustment of antibiotic treatment.

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496 **Table 1: Strains of *S. pneumoniae* used in this study**

Strain	MLST	Serotype	MIC penicillin [µg/ml]*	Frequency of cells with higher penicillin resistance†	Heteroresistance class	Reference or source
Wild types						
R6	128	nt	0.023	-	-	(34, 47)
Spain$^{23F}_{2349}$	81	23F	2.0	10^{-4}-10^{-3}	II	(8, 34)
Spain^{6B}-2	90	6B	1.5	-	-	(16)
***pbp*$_{2349}$ transformants**						
R6::*pbp1a*$_{2349}$	128	nt	0.032	-	-	This study
R6::*pbp2b*$_{2349}$	128	nt	0.032	-	-	This study
R6::*pbp2x*$_{2349}$	128	nt	0.032	10^{-4}-10^{-3}	II	(34)
R6::*pbp2b1a*$_{2349}$	128	nt	0.032	-	-	This study
R6::*pbp2b2x*$_{2349}$	128	nt	0.094	10^{-4}-10^{-1}	II	This study
R6::*pbp2b2x*$_{2349}$ HOM*1	128	nt	0.094	10^{-5}-10^{-1}	II	This study
R6::*pbp2b2x*$_{2349}$ HOM*2	128	nt	0.125	10^{-4}-10^{-2}	II	This study
R6::*pbp2b2x*$_{2349}$ HOM*3	128	nt	0.125	10^{-4}-10^{-2}	III	This study
R6::*pbp2b2x*$_{2349}$ HOM*3p	128	nt	0.094	10^{-4}–10^{-1}	II	This study
R6::*pbp2x1a*$_{2349}$	128	nt	0.032	10^{-5}-10^{-4}	III	(34)
R6::*pbp2x1a*$_{2349}$ HOM*1	128	nt	0.064 (0.25)‡	10^{-5}-10^{-2}	II	This study
R6::*pbp2x1a*$_{2349}$ HOM*2	128	nt	0.064 (0.5)‡	10^{-4}-10^{-2}	II	This study
R6::*pbp2x1a*$_{2349}$ HOM*3	128	nt	0.094 (0.38)‡	10^{-5}-10^{-2}	II	This study
R6::*pbp2x1a*$_{2349}$ HOM*3p	128	nt	0.047 (0.5)‡	10^{-4}-10^{-2}	III	This study
R6::*pbp2x2b1a*$_{2349}$	128	nt	1.5	10^{-5}-10^{-3}	II	This study
R6::*pbp2b*$_{2349}$*pbp2x*$_{6B}$	128	nt	0.125	10^{-5}-10^{-1}	II	This study
R6::*pbp1a*$_{2349}$*pbp2x*$_{6B}$	128	nt	0.125	10^{-5}-10^{-4}	III	This study
***ciaR* disruption mutants (Δ*ciaR*)**						
R6*ciaR*::*aad9*	128	nt	0.012	-	-	(48)
R6*pbp2x*$_{2349}$*ciaR*::*aad9*	128	23F	0.012	10^{-2}-10^{-4}	II	This study

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498 *MIC as determined by E-test, which was performed three times. Values were within one doubling dilution.

499 †Frequency of subpopulations with higher penicillin resistance levels as determined by PAP

500 ‡MIC for subpopulaton growing in the zone of inhibition of E-test

501 nt = non typeable

502

503

Figure legends

Figure 1: Population analysis profiles (PAP) for single, double and triple transformants.

PAP for penicillin of *S. pneumoniae* penicillin-binding protein (*pbp*) transformants harbouring one resistance gene, *pbp2b*, *pbp2x* or *pbp1a*, of heteroresistant strain Spain$^{23F}_{2349}$ in the background of the non-heteroresistant strain R6 compared to R6 wild-type (A), double-transformants with two *pbp*s$_{2349}$ compared to R6 (B) and a triple-transformant containing all three *pbp* genes in R6 background compared to Spain$^{23F}_{2349}$ and R6 wild-type (C). The concentration of penicillin G used to select subpopulations with higher penicillin resistance levels is shown against the frequency of bacteria able to grow at that concentration. Representatives of three independent experiments are shown. Replicates are reported in supplementary information (Figures S1 & S2).

Figure 2: Influence of *pbp2x* gene sequence, genetic background, and CiaRH system on heteroresistance

PAP for penicillin of transformants harbouring *pbp2b* of heteroresistant strain Spain$^{23F}_{2349}$ plus *pbp2x* of non-heteroresistant strain Spain^{6B}-2 (A) and *pbp1a*$_{2349}$ plus *pbp2x*$_{6B}$ (B) in the background of non-heteroresistant strain R6 are shown. Mutants with silenced CiaRH system through disruption of *ciaR* in mutants of R6::*pbp2x*$_{2349}$ and R6 compared to the original strains are shown, too (C). Replicates are reported in Figure S4.

Figure 3: PAP for HOM* strains of double-transformants with heteroresistance to penicillin.

HOM*1, HOM*2 and HOM*3 stand for derivatives of the respective strains obtained by selection of single colonies during successive PAP experiments. HOM*3p is progeny of HOM*3 which has been passaged 15 times on selection- free media.

Single-colonies were picked from plates with highest or second-highest penicillin concentration showing bacterial growth. Original and progeny of double-transformants harbouring *pbp2b* and *pbp2x* of heteroresistant strain Spain$^{23F}_{2349}$ in R6 background (A) and *pbp2x* and *pbp1a* in R6 background (B) are shown. Additional HOM* lineages are reported in Figure S5.

Figure 4: Protein and mRNA expression levels between R6::*pbp2b2x*$_{2349}$ original transformant, HOM*1 and HOM*3 progeny.

Shotgun protein expression profiles were compared between R6::*pbp2b2x*$_{2349}$ original transformant and HOM*3 progeny (A). Relative mRNA expression levels of *pstS* and *pstB* of R6::*pbp2b2x*$_{2349}$ and its HOM*1 and HOM*3 progeny are shown (B). Penicillin-binding protein quantities were compared for R6::*pbp2b2x*$_{2349}$ original transformant and HOM*3 progeny stained with BocillinFL after separation on SDS-PAGE (left lanes), and total protein amount staining with Coomassie brilliant blue (right lanes) (C). mRNA expression of *pbp2x and pbp2b* genes were compared between R6::*pbp2b2x*$_{2349}$ original transformant and HOM*3 progeny (D). Gene expression is displayed as the value relative to that of the isolate with the lowest expression, after normalization using 16S RNA gene expression. Means of three independent experiments are shown. Error bars indicate SEM. MW ; Molecular weight marker. WT; wild type. HOM*; highly resistant subpopulation

**** $p \leq 0.0001$, *** $p \leq 0.001$.