Heteroresistance to penicillin in *Streptococcus pneumoniae*

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Heteroresistance to β -lactam antibiotics has been mainly described for staphylococci, for which it complicates diagnostic procedures and therapeutic success. This study investigated whether heteroresistance to penicillin exists in *Streptococcus pneumoniae*. Population analysis profile (PAP) showed the presence of subpopulations with higher penicillin resistance in four of nine clinical pneumococcal strains obtained from a local surveillance program (representing the multiresistant clones ST179, ST276, and ST344) and in seven of 16 reference strains (representing the international clones Spain^{23F}-1, Spain^{9V}-3, Spain¹⁴-5, Hungary^{19A}-6, South Africa^{19A}-13, Taiwan^{23F}-15, and Finland^{6B}-12). Heteroresistant strains had penicillin minimal inhibitory concentrations (MICs) (for the majority of cells) in the intermediate- to high-level range (0.19–2.0 μ g/ml). PAP curves suggested the presence of subpopulations also for the highly penicillin-resistant strains Taiwan^{19F}-14, Poland^{23F}-16, CSR^{19A}-11, and CSR¹⁴-10. PAP of bacterial subpopulations with higher penicillin resistance showed a shift toward higher penicillin-resistance levels, which reverted upon multiple passages on antibiotic-free media. Convergence to a homotypic resistance phenotype did not occur. Comparison of two strains of clone ST179 showed a correlation between the heteroresistant phenotype and a higher-penicillin MIC and a greater number of altered penicillin-binding proteins (PBP1a, -2b, and -2x), respectively. Therefore, heteroresistance to penicillin occurs in international multiresistant clones of *S. pneumoniae*. Pneumococci may use heteroresistance to penicillin as a tool during their evolution to high penicillin resistance, because it gives the bacteria an opportunity to explore growth in the presence of antibiotics before acquisition of resistance genes.

Penicillin resistance has emerged in *Streptococcus pneumoniae* within a few decades after the introduction of penicillin and has spread successfully worldwide. A relatively small number of resistant clones have been mainly responsible for the current international resistance epidemiology (1, 2). Determinants of pneumococcal penicillin resistance are mutations in high-molecular-weight class A and B penicillin-binding proteins (PBP), which probably accumulate in commensal streptococci and are then transformed into pneumococci (3, 4). There is increasing evidence that auxiliary genes are needed for the expression of pneumococcal penicillin resistance, such as the *murMN* genes and the *ciaH/R* two-component system (5–8). For genetic and statistical reasons, it is unlikely that the different resistance components are acquired during a single transformation or mutation event. However, it is unknown whether there is a defined chronological order or whether single components can provide a selection advantage to the bacterial cell on the way to resistance.

Heteroresistance may play a role in this evolutionary process. The term has not yet been clearly defined, but it is usually understood as the presence of one or several bacterial 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. Most studies have concentrated on heteroresistance to methicillin and vancomycin in staphylococci (9–15). There are some reports of other pathogens, such as heteroresistance to rifampicin in mycobacteria (16), to

vancomycin in enterococci (17), to colistin in *Acinetobacter* spp (18), and to fluconazole in *Cryptococcus neoformans* (19).

Heteroresistance creates clinical and diagnostic problems, but it is also intriguing from an evolutionary standpoint. Heteroresistance may give the microorganism the opportunity to explore growth at higher antibiotic concentrations without paying the fitness costs that may be associated with the acquisition of resistance genes, such as altered PBP genes (20). Heteroresistance may therefore serve as a tool used by bacteria during evolution to resistance.

In this study, we searched for heteroresistance to penicillin in *S. pneumoniae*. We were motivated by a phenomenon observed during resistance testing of clinical pneumococcal isolates collected within a nationwide surveillance program (21). Upon determination of the penicillin MIC by the Etest method (AB Biodisk, Solna, Sweden), some strains exhibited an inner zone of hemolysis but no visible bacterial growth. We hypothesized that this inner hemolysis zone may indicate the growth of a subpopulation of bacteria with higher resistance levels.

Results

Antibiotic Susceptibility Testing. Penicillin MICs determined by Etest of the study strains are shown in Table 1 (22, 23). Interpretation of penicillin Etests for strains 106.44, 110.58, 208.39, and 304.80 was ambiguous because of an inner zone of hemolysis without visible bacterial growth of appreciable diameter (>5 mm) (Fig. 1A). For example, for strains 208.39 and 304.80, interpretation of the Etest based on the bacterial lawn yielded an MIC of 0.008 μ g/ml, whereas the MIC was 0.75 μ g/ml based on the width of the hemolysis zone. For strains 110.58 and 106.44, MIC values based on the bacterial lawn were 0.012 and 0.5 μ g/ml, respectively. However, the hemolysis zone indicated a MIC of 0.19 and 1.0 μ g/ml, respectively. MIC values obtained from macrobroth dilution were even higher than the MIC read from the hemolysis zone of the Etest; it was 1.29 μ g/ml for strain 208.39, 1.44 μ g/ml for strain 304.80, 0.75 μ g/ml for strain 110.58, and 1.0 μ g/ml for strain 106.44. These observations suggested the presence of subpopulation(s) with higher resistance level(s) in strains 106.44, 110.58, 208.39, and 304.80. Subcultures taken from the hemolysis zone and inoculated on Columbia sheep blood agar (CSBA) plates yielded bacterial growth in all four strains. MIC values obtained from these subcultures were comparable to the values for the majority of the population (0.75 μ g/ml for strain 208.39, 0.75 μ g/ml for strain

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Abbreviations: MIC, minimal inhibitory concentration; MLST, multilocus sequence typing; PAP, population analysis profile; PBP, penicillin-binding protein; PFGE, pulsed-field gel electrophoresis; CSBA, Columbia sheep blood agar.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EF989125–EF989160).

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Table 2. Population analysis profile for penicillin resistance of *S. pneumoniae* strains

Strain	MLST type	MIC, [†] $\mu\text{g/ml}$	Frequency of cells with higher resistance [‡]	Highest penicillin concentration with bacterial growth, [§] $\mu\text{g/ml}$
111.46	177	0.064	None	0.05
207.41	179	0.064	None	0.08
202.47	177	0.094	None	0.08
202.58	305	0.032	None	0.04
208.39 [¶]	276	0.75	10^{-3} to 10^{-5}	0.75
HOM*1		1.0	10^{-3} to 10^{-6}	1.75
HOM*1p		1.0	10^{-3} to 10^{-5}	1.25
HOM*2		2.0	10^{-3} to 10^{-5}	1.50
HOM*3		3.0	10^{-3} to 10^{-5}	2.4
304.80 [¶]	179	0.75	10^{-3} to 10^{-5}	1.0
HOM*1		1.0	10^{-2} to 10^{-4}	1.5
HOM*1p		0.75	10^{-3} to 10^{-6}	1.25
HOM*2		4.0	10^{-3} to 10^{-5}	2.1
HOM*3		2.0	10^{-3} to 10^{-5}	2.1
106.44 [¶]	344	1.0	10^{-4} to 10^{-5}	1.0
HOM*1		1.0	10^{-4} to 10^{-5}	1.5
HOM*1p		1.0	10^{-2} to 10^{-5}	0.9
HOM*2		3.0	10^{-2} to 10^{-3}	1.5
HOM*3		3.0	10^{-2} to 10^{-4}	1.5
110.58	344	0.19	10^{-6}	0.6
306.75	1560	0.064	None	0.05
Spain ^{23F} -1	81	2.0	10^{-2} to 10^{-3}	2.0
Spain ^{6B} -2	90	1.5	None	1.25
Spain ^{9V} -3	156	2.0	10^{-3} to 10^{-6}	2.1
Hungary ^{19A} -6	268	1.0	10^{-3} to 10^{-5}	2.5
South Africa ^{19A} -7	75	0.38	None	0.325
South Africa ^{6B} -8	185	0.25	None	0.325
England ¹⁴ -9	9	0.032	None	0.03
CSR ¹⁴ -10	20	12	None	14.0
CSR ^{19A} -11	175	12	None	8.0
Spain ¹⁴ -5	18	2	10^{-5}	3.5
Finland ^{6B} -12	270	0.75	10^{-3}	1.0
South Africa ^{19A} -13	41	3.0	10^{-3}	3.0
Taiwan ^{19F} -14	236	8.0	None	6.0
Taiwan ^{23F} -15	242	1.5	10^{-3} to 10^{-6}	2.0
Poland ^{23F} -16	173	16	None	8.0
Tennessee ^{23F} -4	37	0.125	None	0.1
D39	128	0.023	None	0.02
R6	128	0.026	None	0.02
QK25/010641	Not determined	0.38	None	0.32

[†]MIC determined by Etest.

[‡]Frequency of subpopulations with higher penicillin-resistance levels as determined by population profile analysis (see also Figs. 2 and 3).

[§]The highest penicillin concentration at which bacterial growth could be observed during population profile analysis.

[¶]HOM* strains were generated by propagation of cells from subpopulations with higher penicillin-resistance level as explained in *Materials and Methods*.

Stability of Heteroresistance to Penicillin. At least three repetitions of PAP for each strain starting from the frozen bacterial stock documented a remarkable stability of the heteroresistant phenotype [for details, see supporting information (SI)]. The resistance behavior of subpopulations with higher resistance levels was analyzed for strains 208.39, 304.80, and 106.44. Single colonies were picked from PAP plates with the highest or second-highest penicillin concentrations showing bacterial growth and subjected to PAP immediately or after 10 passages on antibiotic-free agar plates. The resulting progeny strains were called HOM*1 and HOM*1p, in reminiscence of the work done in staphylococci (11). HOM*1 generations showed a shift of the PAP curve to slightly higher penicillin concentrations, but the shape or class of the PAP curve was essentially preserved (Fig. 3). Passage of HOM*1 strains 10 times without antibiotics shifted the PAP curve back to the range of penicillin concentrations of the parental strain for 304.80 and 106.44 but not for strain 208.39-HOM*1 (Fig. 3).

PAP of HOM*2 and HOM*3 strains (generated from HOM*1 and HOM*2 strains, respectively) showed the same trend as observed for HOM*1 strains, with a gradual shift of the curves toward higher penicillin concentrations. Again, the overall shape or class of the curve was maintained, i.e., HOM* strains did not convert to a homogeneous resistance profile (Fig. 3). Interestingly, for HOM*2 and HOM*3 strains, satellite colonies appeared during Etest for penicillin (Fig. 1B).

PBP Profiles in HOM* Strains. To explore whether HOM* derivatives expressed the same PBP genes as their parent strains, the transpeptidase region of *pbpl*a, -2b, and -2x was sequenced for the strains 208.39, 304.80, 106.44, and 110.58 (GenBank accession nos. EF989125–EF989160). Numerous attempts to obtain sequences for the *pbpl*a fragment downstream of the conserved motif II for strain 110.58 and its HOM* derivatives were unsuccessful. Analysis of all other sequences showed that HOM*

Fig. 2. PAP for *S. pneumoniae* strains (Table 1). The x axis indicates the penicillin concentration in micrograms per milliliter used to select supopulations with higher penicillin-resistance levels, and on the y axis, the frequency of bacterial cells is given as the logarithm to the base 10 of cfu per milliliter. (A) PAP for *S. pneumoniae* strains collected in Switzerland and laboratory strains (see also Table 1). (B) PAP for *S. pneumoniae* strains representative of international clones (see also Table 1). (C) PAP for *S. pneumoniae* strains representative of international clones (see also Table 1).

derivatives carried the same *pbp1a*, *-2b*, and *-2x* genes as their parent strains.

Patterns of radiolabeled PBPs also confirmed that the HOM* strains of the heteroresistant strains 208.39, 304.80, 106.44, and 110.58 expressed the same PBPs as their parent strains (for details, see SI).

Colony Size. Strains with heteroresistance to penicillin exhibited a picture of varying colony size when grown on CSBA plates with or without penicillin, as demonstrated for strain 304.80 in Fig. 4. The range of colony diameters was wider for strain 304.80 than strain D39 with or without the presence of penicillin in agar plates. However, penicillin MIC values and PAP did not differ between small and large colonies (for details, see SI).

Phylogenetic Relationships Among Swiss Strains. Molecular typing was performed for the Swiss pneumococcal strains (Table 3). Some of these data have been reported (22, 23).

The serotype 19F strains 111.46, 202.47, 207.41, and 304.80 were phylogenetically related, because they all belong to the pulsed-field gel electrophoresis (PFGE) clone H (22, 24), and they belong to multilocus sequence typing (MLST) 177 or 179, which differ for the

Fig. 3. PAP for the HOM* strains of three Swiss strains 208.39, 304.80, and 106.44 with heteroresistance to penicillin. HOM*1, HOM*2, and HOM*3 stands for derivatives of the respective strains obtained by selection of single colonies during successive PAP experiments. Colonies were selected from the plate with the highest or second-highest penicillin concentration showing bacterial growth. HOM*1p stands for a HOM*1 strain that has been passaged 10 times on CSBA plates without antibiotics before repeating PAP. The *x* axis indicates the respective penicillin concentration in micrograms/milliliters used to select resistant subpopulations; on the *y* axis, the frequency of bacterial cells is given as the logarithm to the base 10 of cfu per milliliter.

gki allele only (type 40 instead of type 4). By PFGE, strains 207.41 and 304.80 differed by two bands (data not shown). Interestingly, of these four related strains, only strain 304.80 exhibited heteroresistance to penicillin, but it also showed the highest penicillin MIC and the greatest number of altered *pbp* genes (Table 3).

Serotype 19F strain 208.39 with heteroresistance to penicillin was a member of the international resistant clone ST276 (25).

Strains 110.58 and 106.44 belonged to the nonencapsulated clone ST344, but their PFGE patterns differed by two bands (data not shown). Also, they showed mutated *pbp1a*, -2b, and -2x genes and differed in their gene sequence between each other (Table 3). Both strains exhibited heteroresistance to penicillin, but strain 110.58 with lower penicillin MIC had only one subpopulation at low frequency (Fig. 2A).

All four Swiss strains with heteroresistance to penicillin had mutations in all three *pbp* genes analyzed. Intriguingly, three of the four strains (208.39, 304.80, and 106.44) showed a high sequence homology for the *pbp2x* gene, and strains 304.80 and 106.44 also shared the *pbp1a* gene sequence.

Fig. 4. *S. pneumoniae* strains with heteroresistance to penicillin exhibited visible heterogeneity of bacterial colony size when grown on CSBA plates with or without penicillin. This is demonstrated by comparing colony size diameters between the laboratory strain D39 and strain 304.80 grown on agar plates without antibiotics (A) and plates containing 0.02 µg/ml penicillin for strain D39 and 0.5 µg/ml penicillin for strain 304.80 (B). The x axis indicates the colony diameter in millimeters. The y axis gives the proportion of colonies with the respective colony diameter.

Discussion

Heteroresistance describes the existence of one or several subpopulations of bacterial cells with higher resistance levels than the majority of cells in a population profile analysis (26). Most observations on heteroresistance reported in the literature concern staphylococci resistant to methicillin, vancomycin, and/or inhibitors of teichoic acid synthesis (9–15). This study provides evidence for the existence of heteroresistance to penicillin in *S. pneumoniae*.

The results presented here for *S. pneumoniae* are well in line with reports on heteroresistance to methicillin in staphylococci. Pneumococcal subpopulations with higher resistance levels occurred at expected frequencies (10^{-3} to 10^{-6}). Other characteristics of heteroresistance, such as heterogeneity in colony size and growth of satellite colonies in the Etest, could also be observed. Similar to the findings for staphylococci, PAP results were remarkably stable for a given strain (11, 14, 27). *Staphylococcus aureus* with heteroresistance to methicillin have been categorized into four classes based on the shape of the PAP curves (11). The *S. pneumoniae* strains with heteroresistance to penicillin described here showed mainly a class II pattern, which stands for the existence of several subpopulations with different penicillin resistance levels. Only few strains fell into class III with one subpopulation only.

In methicillin-resistant *S. aureus*, propagation of highly resistant subpopulations in the presence of methicillin induced conversion from heterotypic to homotypic resistance (11, 14). Repeated propagation of HOM* strains for *S. pneumoniae* with heteroresistance to penicillin also showed a gradual increase in penicillin-resistance levels but only a trend of converting to a homotypic resistance pattern. Upon passage of HOM*1, strains in antibiotic-free strains tended to return to the penicillin-resistance phenotype of their parent strain.

All pneumococcal strains with heteroresistance to penicillin identified in this study belonged to well characterized international multiresistant clones (1, 25, 28). These strains had penicillin MIC levels for the majority of bacterial cells ranging between 0.19 and 2.0 µg/ml. That PAP of strains with very high penicillin MICs were only suggestive of heteroresistance to penicillin, although they showed satellite colonies in Etest, may be explained by a biological limit set to the pneumococcus for the highest-attainable MIC value.

The mechanisms involved in staphylococcal heteroresistance to methicillin are complex, and a considerable number of candidate genes have been implicated (9, 10, 12, 13, 15). This is likely also the case for heteroresistance to penicillin in *S. pneumoniae*. Three of the four Swiss strains with heteroresistance to penicillin shared the same *pbp2x* gene sequence but, in analogy to heteroresistance to methicillin in staphylococci, it seems unlikely that single PBP variants alone should be responsible for heteroresistance to penicillin. In preliminary experiments, we were unable to transfer heteroresistance to penicillin to other pneumococcal strains by transformation with the *pbp2x* gene of strain 304.80 (data not shown). Therefore, it seems more likely that known and/or unknown auxiliary resistance genes in concert with PBP variants are responsible for heteroresistance to penicillin in *S. pneumoniae*.

Table 3. Molecular typing of selected strains of *S. pneumoniae*

Strain	Serotype	MLST	Heteroresistance to penicillin	Penicillin MIC, * µg/ml	RFLP of PBP [†]		
					2x	2b	1a
111.46	19F	177	No	0.064	A	R6	R6
202.47	19F	177	No	0.094	Bs	R6 s	As
207.41	19F	179	No	0.064	A	R6	R6
304.80	19F	179	Yes	0.75	Cs	As	Bs
208.39	19F	276	Yes	0.75	Cs	R6s [‡]	Ds
110.58	Nt	344	Yes	0.19	Bs	Bs	Cs
106.44	Nt	344	Yes	1.0	Cs	Cs	Bs
D39	2	128	No	0.023	R6s	R6 s	R6s
R6	Nt	128	No	0.026	R6s	R6 s	R6s

*MIC determined by Etest.

[†]RFLP of PBP: RFLP profile analysis of PBP genes 1a, 2b, and 2x; R6 means that the RFLP pattern for this strain was identical to, and A–D means the pattern showed one band or more difference from, the pattern observed for the laboratory strain R6; “s” indicates that the RFLP result was confirmed by DNA sequencing; sequences were called “identical” if nucleotide homology reached >96.5%.

[‡]The *pbp2b* DNA sequence in strain 208.39 revealed a fragment of 65-bp length immediately upstream of the conserved SXN motif II with 31% heterogeneity compared with the corresponding sequence in strain R6.

Heteroresistance to penicillin may be used by *S. pneumoniae* during evolution to higher-level penicillin resistance. This study included four phylogenetically related pneumococcal strains belonging to PFGE clone H or MLST ST177 and ST179, but only the strain with highest-penicillin MIC level and the greatest number of altered PBP genes was heteroresistant to penicillin. Earlier reports have described the plasticity of this clone in terms of penicillin-resistance levels and acquisition of *pbp* gene fragments (22, 24, 28). In addition, the study analyzed two nonencapsulated strains of MLST ST344. Both exhibited heteroresistance to penicillin. However, in the strain with lower-penicillin MIC (strain 110.58), subpopulations with higher resistance levels occurred at lower frequency (10^{-6}). It will be interesting to extend such analyses to other international pneumococcal clones.

In conclusion, this study provides evidence that heteroresistance to penicillin exists in *S. pneumoniae* and can be found in members of international multiresistant clones. We speculate that *S. pneumoniae* uses heteroresistance to penicillin during evolution to higher penicillin resistance. Heteroresistance may allow bacterial cells to explore growth at higher-penicillin concentrations without paying the fitness costs associated with the acquisition of new *pbp* gene fragments (20).

Materials and Methods

Bacterial Strains and Culture Conditions. Bacterial strains used for this study are presented in Table 1. The local Swiss strains of serotype 19F were part of a recent analysis of 108 clinical nasopharyngeal isolates of serotype 19F with an oxacillin disk diameter of <20 mm (1- μ g disk) (22). Some of the nontypable isolates (106.44 and 110.58) have also been reported (22, 23). Reference strains for the international clones were kindly provided by Ralf Reinert (National Reference Center for Pneumococcus, Aachen, Germany). Control strains included the laboratory strain D39 (serotype 2), its spontaneous nonencapsulated derivative R6, and strain ATCC49619 (serotype 19F) (29).

The local Swiss pneumococcal strains were originally isolated from nasopharyngeal swabs from patients with acute respiratory tract infection (21, 28) and were stored after one *in vitro* passage at -80°C by using Protect bacterial preservers (Technical Service Consultants, Heywood, U.K.). For culture, bacteria were grown on CSBA plates at 37°C in a 5% CO_2 -enriched atmosphere or in brain-heart infusion broth (Becton Dickinson, le Pont de Claix, France) containing 5% FCS (Biochrom, Berlin, Germany) at 37°C in ambient air.

Molecular Typing. MLST was done as described (30). The methods for PFGE, RFLP, and sequence typing of the genes encoding for PBP1a, -2b, and -2x have been reported (22). Membrane-enriched protein fractions were prepared, and radiolabeled PBPs were detected as described (31).

Antibiotic Susceptibility Testing. MIC were determined by the Etest method (AB Biodisk, Solna, Sweden) for penicillin and vancomycin. Macrobroth dilution was done for penicillin [Sigma-Aldrich (Buchs, Switzerland), P8721; 10 million units, Lot123K05211; Fluka, Buchs, Switzerland] in cation-adjusted Mueller-Hinton broth (Becton Dickinson), according to Clinical and Laboratory Standards Institute guidelines (32).

PAP. PAP was performed for resistance to penicillin and vancomycin according to the method described by Wootton *et al.* (26), with some adaptations. Briefly, bacteria were streaked out on CSBA plates and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. An overnight culture was prepared by inoculating 3–10 colonies into 5 ml of brain heart infusion (BHI) containing 5% FCS in 15-ml tubes (Sarstedt, St. Gallen, Switzerland). The tubes were placed in a 37°C water bath for 9 h. One hundred microliters of the overnight culture was subcultured in 5 ml of BHI with 5% FCS and was grown to midlog phase (OD_{600} 0.7 encapsulated, OD_{600} 0.5–0.6 unencapsulated strains). Dilutions of this culture of 10^{-2} to 10^{-4} and 10^{-6} in PBS (pH 7.4) were prepared, and 100 μl was spiral-plated on Müller-Hinton broth (MHB) agar plates (Bio-Mérieux, Geneva, Switzerland) with 5% sheep blood containing penicillin concentrations ranging from 0 to 18 $\mu\text{g}/\text{ml}$ or vancomycin (vancomycin HCl, Sigma V2002, Lot 015K0825, Fluka) (concentrations 0.025, 0.125, 0.25, and 0.5 $\mu\text{g}/\text{ml}$). A dilution of the culture of 10^{-6} was spiral-plated onto a MHB plate with no antibiotic for determination of colony count. Colonies were counted by eye after 48 h of incubation at 37°C in 5% CO_2 .

PAP for HOM*. Single colonies were selected from agar plates containing the highest or second-highest penicillin concentration at which bacterial growth was detectable. These colonies, called HOM*1 (11), were subcultured once (at a maximum, twice if necessary for sufficient growth) on CSBA plates without antibiotics and frozen at -80°C until PAP analysis. This procedure was repeated for HOM*1 strains yielding the HOM*2 generation and for HOM*2 strains generating HOM*3 strains. To test the stability of penicillin resistance, HOM*1 strains were subcultured for 8–10 passages on CSBA plates without antibiotics before PAP.

Determination of Colony Size. Colony size was determined during PAP experiments from CSBA plates after incubation for 24 h. The diameter of each colony was measured by using a stereomicroscope with an integrated 0.1-mm scale (Leica GZ4, Heerbrugg, Switzerland) at $\times 10$ magnification.

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