

Persistence of Resistance Plasmids Carried by Beta-Hemolytic *Escherichia coli* When Maintained in a Continuous-Flow Fermentation System Without Antimicrobial Selection Pressure

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Abstract

It is thought that antimicrobial resistance imposes a fitness cost on bacteria, so that a reduction in antimicrobial use may reduce the incidence of resistant bacteria. The objectives of the present study were to determine (1) whether multidrug resistant (MDR) *Escherichia coli* field strains with different plasmid profiles show disparate plasmid loss when grown over time without selection pressure; (2) whether the number of plasmids present in the cell affects growth. Nine β -hemolytic *E. coli* strains from swine ($n=8$) and cattle ($n=1$) were grown in separate continuous-flow vessels for 36 days without antimicrobial selection. Populations were enumerated on brain heart infusion agar and brain heart infusion agar with tetracycline on days 2, 5, 8, 15, 22, 29, and 36. Growth rates, plasmid profiles and susceptibility profiles of the strains were compared, and day 36 isolates ($n=40$, five for each MDR strain) were compared with their corresponding day 0 strains. Plasmid content of the nine field strains ranged from zero to eight with sizes from 3.2 to 165 kb. Changes in susceptibility profiles of day 36 isolates were observed among 20% (8 of 40) of the isolates. MDR *E. coli* largely maintained their original plasmid profiles, replicon types, and susceptibility profiles over 36 days of continuous culture. There was no significant difference in maximum specific growth rate among strains when compared with the plasmid-free strain or when day 36 isolates were compared with their own day 0 strain. This suggests that there is little fitness cost in the maintenance of multiple plasmids of various sizes under the conditions of this study. Other strategies rather than merely reducing antimicrobial usage are needed to combat the emergence of MDR bacteria.

Introduction

ACQUISITION, MAINTENANCE, and expression of antimicrobial resistance imposes a fitness cost to the bacterium (Schrage and Perrot, 1996; Andersson and Hughes, 2010). Although this fitness cost may initially be substantial, compensatory mechanisms ultimately develop to reduce the cost of maintaining resistance (Björkman and Andersson, 2000). This has been well studied for chromosomal mutations (Schrage and Perrot, 1996; Schrage *et al.*, 1997; Reynolds, 2000), but less is known about compensation for plasmid acquired resistance (Lenski *et al.*, 1994; Björkman and Andersson, 2000; Dahlberg and Chao, 2003). Several factors may affect the reversal of resistance resulting from plasmid acquisition in bacteria (Johnsen *et al.*, 2009). The high rate of conjugation in a bac-

terial population may offset the rate of plasmid loss by generation of plasmid-free daughter cells during segregation (Lundquist and Levin, 1986; Simonsen *et al.*, 1990; Dionisio *et al.*, 2002).

Selective pressure exerted by the use of antimicrobials in both human and animal populations over the past several decades has led to the emergence of multidrug resistant (MDR) bacterial populations that are resistant to many commercially available drugs (Levin, 2001). This is an increasing threat to both human and animal health. Attention has been focused on mobile genetic elements such as plasmids that carry multiple resistance genes, because the horizontal transfer of plasmids allows the rapid dissemination of MDR to susceptible populations (Barlow, 2009). The gastrointestinal metagenomic/metaplasmidic reservoir is of concern, because

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many common antimicrobials, used in both human and animal applications, are afforded the opportunity to affect this commensal population (Poole *et al.*, 2004).

Previous studies have determined that foodborne pathogenic bacteria can carry multiple antimicrobial resistance plasmids (Johnson *et al.*, 2007; Fricke *et al.*, 2009; Poole *et al.*, 2009). However, little is still known about the fitness cost incurred by bacteria from the maintenance of multiple plasmids. This study used continuous-flow fermentation devices that have been previously used to model the gastrointestinal tracts of poultry and swine (Nisbet *et al.*, 1993; Genovese *et al.*, 2000) to assess plasmid loss over time as well as the fitness costs attributed to the number of plasmids harbored by selected *Escherichia coli* strains. Characterizing the plasmid profiles and understanding the fitness costs associated with maintenance of multiple plasmids may allow targeted animal production practices to reduce the number of MDR strains.

Materials and Methods

Bacterial isolates

The *E. coli* 1572 (was provided by Dr. Ken Bischoff, USDA/ARS/NCAUR, Peoria, IL) was plasmid free and pan-susceptible to antimicrobials, and for this study was referred to as 1572^s. Strain 1572^s was used as a plasmid negative control strain for polymerase chain reaction (PCR)-based replicon testing (PBRT), plasmid analysis, log-phase growth studies, and continuous-flow growth studies. Nalidixic acid and rifampicin resistant *E. coli* 1572^{nr}, *E. coli* JM109F^{nr}, and *E. coli* Dh5 α F^{nr} have been previously described as recipient strains for conjugation studies (Poole *et al.*, 2009). *E. coli* strain 6-3-155b (155 in this study) was provided by Dr. Guy Loneragan, Texas Tech University, Lubbock, TX. *E. coli* strains 831, 834, 840, 843, 1548, 1562, and 1568 from swine have been previously described (Bischoff *et al.*, 2002, 2005; Beier *et al.*, 2005). These isolates were chosen due to plasmid content heterogeneity (Table 1). Bischoff *et al.* (2005) determined that the chloramphenicol resistance gene *cmlA* was present in combination with class I integrons on plasmids ranging from 130 to 270 kb for strains 831, 843, 1548, and 1562. Strain 1548 has been used *in vitro* and *in vivo* to study conjugative transfer (Poole *et al.* 2006, 2009). The A/C plasmid has been sequenced and is known to carry *bla*_{CMY-2} that confers resistance to cephalosporins (Welch *et al.*, 2007). Bacterial conjugations

were done at 37°C, using tetracycline for counter selection as previously described (Poole *et al.*, 2009), to determine whether multi-drug resistance phenotypes were transferable to at least one of three recipient strains.

Continuous-flow mono-cultures

E. coli strains 155, 831, 834, 840, 843, 1548, 1562, and 1568 were inoculated in 9 mL of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) and grown overnight at 37°C (day 0). On day 1, each experimental continuous-flow pure culture was initiated by inoculating a fermentation apparatus containing 500 mL anoxic (bubbled with O₂ free CO₂ constantly) Viande Levure broth (10.0 g of tryptose, 5.0 g yeast extract, 5.0 g NaCl, 2.5 g dextrose, 2.4 g beef extract, and 0.6 g L-cysteine per liter, pH 6.0) with 5.0 mL of each overnight pure *E. coli* culture (~10⁸⁻⁹ CFU/mL). Each fermentation apparatus had a dilution rate of 0.0417 h⁻¹ corresponding to a flow rate of 48 mL/h and a vessel turnover time of 24 h (Nisbet *et al.*, 1993).

One milliliter aliquots were serially diluted in phosphate-buffered saline and enumerated in duplicate on brain heart infusion agar (BHIA) (Becton Dickinson) and BHIA with tetracycline (BHIA-T) (32 μ L/mL) on days: 2, 5, 8, 15, 22, 29, and 36 postinoculation. Five isolated colonies for each isolate and each sample day were subcultured from BHIA for phenotypic and genotypic analysis.

Maximum specific growth rate

Maximum specific growth rates (h⁻¹) were measured based on changes in absorbance (Callaway and Russell, 1999) to determine the growth rate of each of the *E. coli* strains on day 1 ($n = 2$ per strain) and day 36 ($n = 2$ for each of the five isolates). Each strain was inoculated into 9.0 mL of TSB and incubated statically overnight at 37°C. Two hundred microliters of each overnight culture was inoculated into 9.8 mL of TSB. Tubes were incubated statically at 39°C, and growth was monitored every 30 min via measurement of changes in optical density at 600 nm using a Spectronic 20D + model spectrophotometer (Spectronic Instruments, Rochester, NY).

Antimicrobial susceptibility phenotypic analysis

Antimicrobial susceptibility profiles of the *E. coli* strains to 15 antimicrobial agents were determined on day 0 and day 36

TABLE 1. PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF DAY 0 *ESCHERICHIA COLI* STRAINS

Strain	Source	PBRT	AR phenotype	P1	P2	P3	P4	P5	P6	P7	P8
1572 ^s	Swine	None	None								
155	Bovine	A/C	AmApFT(Ax)CNSSuTeSxt	165	90						
831	Swine	I1, P, Y	CKSSuTe	120	90	40	5.5	5.2	3.8		
834	Swine	FIB, FIA	ApKSSuTe	90	50						
840	Swine	I1, FIB, FIC	ApCKSSuTe	120	65	60	4.5	4.3			
843	Swine	I1, P	ApCKSuTe	100	90	80	60	6	5	4.9	
1548	Swine	I1, FIB, FIC	CKSSuTeSxt	120	90	80	60	5.2			
1562	Swine	I1, FIB, FIC, P	CKSSuTeSxt	120	90	80	60	5			
1568	Swine	I1, FIB, FIC, HI1	ApGKSSuTe	>165	120	80	60	50	5.5	3.5	3.2

P1–P8 represents size of plasmids (kb) visualized by agarose gel electrophoresis.

PBRT = polymerase chain reaction-based plasmid replicon typing; Antimicrobial resistance phenotypes: Am = amoxicillin–clavulanic acid; Ap = ampicillin; F = cefoxitin; T = ceftiofur; Ax = ceftioxone; C = chloramphenicol; G = gentamicin; K = kanamycin; S = streptomycin; Su = sulfisoxazole; Te = tetracycline; Sxt = trimethoprim–sulfamethoxazole; parenthesis = an intermediate phenotype.

($n = 5$ of each strain). Antimicrobial MICs were determined by broth microdilution according to the Clinical Laboratory Standards Institute (CLSI, 2003). Susceptibility testing was performed as previously described (Poole *et al.*, 2009). Data were interpreted using CLSI breakpoints unless unavailable, then breakpoints from the NARMS 2004 annual report were used (FDA, 2004; CLSI, 2010).

Genotypic analysis

PCR-replicon typing of *E. coli* isolates were performed on day 0 ($n = 2$) and day 36 ($n = 5$) using the method of (Carattoli *et al.*, 2005). Plasmid isolation from *E. coli* isolates was performed using the method of Kado and Liu (1981).

Statistical analysis

Bacterial count data were \log_{10} base transformed before analysis using Microsoft Excel. Maximum specific growth rates, and *E. coli* populations were compared at each time point by Student's *t*-test. *In vitro* fermentations were replicated as described in the text. Time \times treatment interactions were discounted in this model; therefore, only pointwise comparisons were performed. Significance was determined at $p < 0.05$.

Results

Characterization of *E. coli* isolates

Seventeen β -hemolytic *E. coli* isolates were initially characterized by RepPCR (PCR amplification of repetitive chromosomal sequences) (data not shown), susceptibility testing, and PBRT to verify that isolates were genotypically distinguishable and possessed differing plasmid profiles. Isolates 155, 831, 834, 843, 1548, 1562, and 1568 were able to transfer an MDR phenotype to recipient strains, by conjugation. Strain 840 did not produce transconjugants at 37°C with any of the recipient strains when tetracycline was used as the counter selection agent. One plasmid-free and eight multi-plasmid *E. coli* strains were chosen for further study. The results of susceptibility testing, replicon typing, and plasmid characterization for the *E. coli* isolates are shown in (Table 1).

Continuous-flow growth studies

Cell populations of the nine *E. coli* isolates in continuous-flow pure cultures were compared over the 36-day culture period. Isolates: 1572^s (zero plasmid), 155 (two large plasmids), 831 (three large plasmids), 834 (two large plasmids), and 840 (three large plasmids) maintained populations between 10^{7-8} CFU/mL; whereas, isolates 843, 1548, 1562, and 1568 (four–five large plasmids) maintained populations between 10^{6-8} CFU/mL throughout the 36-day culture period (Fig. 1a). The same trend was displayed for cell populations on BHIA-T (Fig. 1b). There was no significant difference ($p < 0.1$) in the total bacterial populations for each time point compared with the tetracycline-resistant population of the same isolate. No growth of 1572^s was seen on BHIA-T. By day 36, all of the isolates except 1548 were present at 10^7 CFU/mL. The population of 1548 was slightly lower at 4.4 and 5.10×10^6 CFU/mL on BHIA and BHIA-T, respectively.

The mean was determined for the four isolates with two to three large plasmids and the four isolates with four to five large plasmids (Fig. 1c). The mean values for these two groups

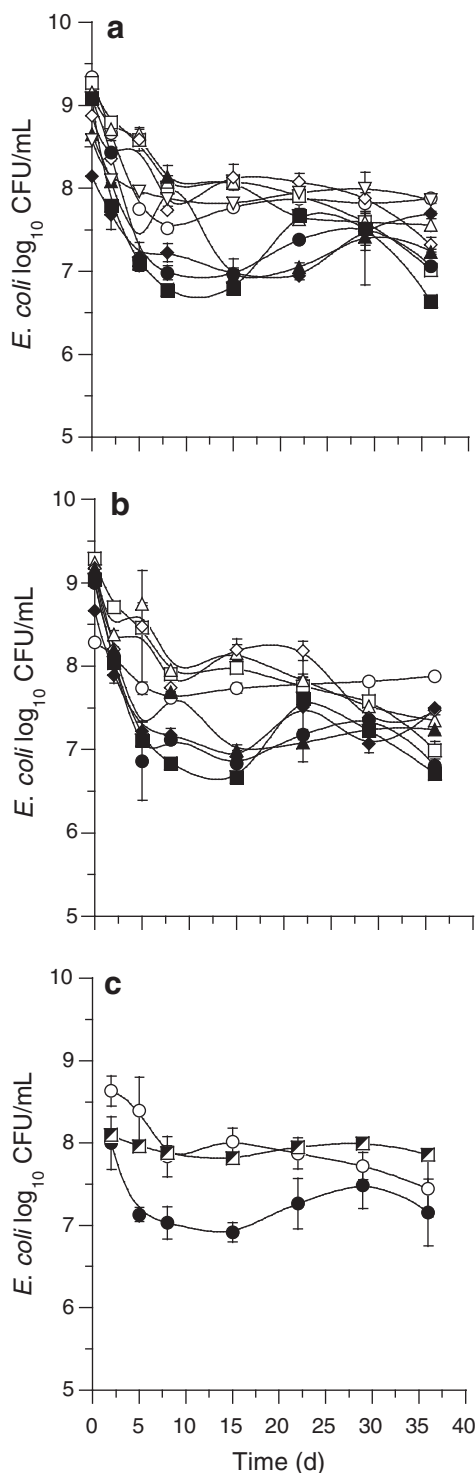


FIG. 1. *Escherichia coli* populations (CFU/mL) in continuous-flow cultures without antimicrobial selection pressure. (a) Plated on brain heart infusion agar (∇) *E. coli* 1572 control; (\circ) *E. coli* 155; (\square) *E. coli* 831; (\triangle) *E. coli* 834; (\diamond) *E. coli* 840; (\bullet) *E. coli* 843; (\blacksquare) *E. coli* 1548; (\blacktriangle) *E. coli* 1562; (\blacklozenge) *E. coli* 1568. (b) Plated on brain heart infusion agar with tetracycline (∇) *E. coli* 1572 control; (\circ) *E. coli* 155; (\square) *E. coli* 831; (\triangle) *E. coli* 834; (\diamond) *E. coli* 840; (\bullet) *E. coli* 843; (\blacksquare) *E. coli* 1548; (\blacktriangle) *E. coli* 1562; (\blacklozenge) *E. coli* 1568. (c) Mean of *E. coli* strains with two to three large plasmids (\circ) 155, 831, 834 and 840; Mean of *E. coli* strains with four to five large plasmids (\bullet) 843, 1548, 1562, 1568 and 1572; plasmid-free *E. coli* 1572 (\blacksquare).

were compared with 1572^S. On day 5, there was a significant difference between the two to three large plasmid group and the four to five large plasmid group at $p < 0.05$.

Replicon genotypes over 36-day study

The replicon content for all replicate isolates, on all time points between day 2 and day 36 ($n = 35$ for each strain), was unchanged as compared with the respective day 0 strains for isolates 155, 831, 834, 840, and 1548. One of the five isolates characterized on day 2 for strain 1562 (designated d 2–4) lacked the P replicon but displayed the same resistance profile as the day0 1562 strain. Isolates 1568 day2–4 and day15–1 were negative for replicon HI1. Isolate 1568 day2–4 displayed the same resistance profile as the day0 strain, but 1568 day 15–1 exhibited resistance only to tetracycline.

Phenotypic and genotypic analysis of day 36 isolates

There was no variation in plasmid or susceptibility profiles for 834, 843, and 1562 day36 isolates as compared with the respective day0 strains. All day36 isolates that displayed variations from day0 are shown in Table 2. Isolates 840 day36–1, 3, 4, and 5 lost resistance to all antimicrobials except tetracycline, but displayed no genotypic changes. Isolate 831 day36–1 acquired resistance to nalidixic acid and trimethoprim-sulfamethoxazole. Both phenotypic and genotypic differences were observed for 155 day36–3 and 840 day36–2. Only genotypic differences in plasmid content were observed for 155 day36–1, 1548 day36–1, and 1568 day36 1–5 isolates. Also, four of the day36 1568 isolates displayed a 75 kb plasmid not apparent in the day0 strain.

Growth studies

Maximum specific growth rates of day0 strains containing plasmids were compared with strain 1572 to determine whether differences based on variations in plasmid content existed. There were no significant differences between any of the plasmid-bearing strains and 1572 at day0. Growth rates for day36 isolates showed that 840 day36–2, 3, and 4 had a significantly higher maximum specific growth rate ($p < 0.05$)

than strain 1572 day36 isolates. When the day0 specific growth rates were compared with their respective day36 isolate specific growth rates, there was no significant difference for any strain.

Discussion

A widely suggested strategy to reduce the environmental prevalence of antimicrobial resistant bacteria is to simply reduce usage of antimicrobial drugs (APUA, 2010). It is thought that antimicrobial resistance incurs a cost to the bacterial cell and in the absence of selection pressure other bacteria in a mixed population, such as the gastrointestinal tract, would out-compete resistant strains (Enne, 2010). This may reduce, but not eliminate, antimicrobial resistant strains, because a rapid rebound of resistant populations has been observed in situations where antimicrobial selection pressure is reapplied. In some cases, there may be no reduction in antimicrobial resistant populations after the decreased usage of a single antimicrobial (Enne *et al.*, 2001; Sundqvist *et al.*, 2010). The data presented here demonstrate the persistence of the MDR phenotype in the absence of selection pressure over a long-term continuous-culture with β -hemolytic *E. coli* isolates from food animals. The bacterial populations in continuous-flow cultures for strains that possessed four or more large plasmids (40 to >165 kb) exhibited a lower initial drop than did strains with two to three large plasmids. This suggests a lesser cost to possession of small plasmids. However, the initial cost observed in the population of strains possessing four or more large plasmids was largely compensated for by day 36. All of the plasmid-bearing strains exhibited a drop in population that is likely due to nongrowth energy dissipation. Since the strains adapted to growth in the continuous-flow vessel, the population rebounded. Adaptation by the strains possessing four or more large plasmids may have required a longer time period. One strain (840) showed significantly higher maximum specific growth rate for three of the day36 isolates (2, 3, and 4) compared with 1572 day36 isolates. Although this was statistically significant, it may not have been biologically significant. The maximum specific growth rate and continuous-flow growth studies suggest that the host bacteria may have

TABLE 2. PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF DAY 36 *ESCHERICHIA COLI* STRAINS THAT DISPLAYED VARIATIONS FROM DAY 0

Strain	PBRT	AbR phenotype	Resistance lost	P1	P2	P3	P4	P5	P6	P7	P8	P9
155 d 36–1	A/C	AmApFT(Ax)CNSSuTeSxt		165	X							
155 d 36–3	A/C	AmApFT(Ax)C(G)NSSuTeSxt		140	90							
831 d 36–1	P, Y, I1	CKNSSuTeSxt		120	90	40	5.5	5.2	3.8			
840 d 36–1	I1, FIB, FIC	Te	ApCKSSu	120	65	60	4.5	4.3				
840 d 36–2	I1, FIB, FIC	Te	ApCKSSu	120	65	60	4.5	4.3	8			
840 d 36–3	I1, FIB, FIC	Te	ApCKSSu	120	65	60	4.5	4.3				
840 d 36–4	I1, FIB, FIC	Te	ApCKSSu	120	65	60	4.5	4.3				
840 d 36–5	I1, FIB, FIC	Te	ApCKSSu	120	65	60	4.5	4.3				
1548 d 36–1	FIC, FIB, I1	CKSSuTeSxt		120	90	60	55	5.2				
1568 d 36–1	FIC, FIB, HI1, I1	ApGKSSuTe		>165	150	80	65	60	5.5	3.5	3.2	75
1568 d 36–2	FIC, FIB, HI1, I1	ApGKSSuTe		>165	150	80	65	60	5.5	3.5	3.2	75
1568 d 36–3	FIC, FIB, HI1, I1	ApGKSSuTe		>165	150	80	65	60	5.5	3.5	3.2	75
1568 d 36–4	FIC, FIB, HI1, I1	ApGKSSuTe		>165	150	80	X	60	5.5	3.5	3.2	
1568 d 36–5	FIC, FIB, HI1, I1	ApGKSSuTe		>165	150	80	65	60	5.5	3.5	3.2	75

Variations from day 0 are shown in bold; an X—represents missing a plasmid band.

adapted to the plasmids or developed compensatory mutations over the course of the 36 day study. It is not known whether adaptation may have occurred in the host animal before isolation of the bacteria in the field or whether it occurred during the course of this study. The conditions used in this study are not representative of the competitive stress in an animal's intestinal tract. Therefore, additional studies in mixed-gut microflora cultures, swine fecal fluid, and swine are currently in progress to determine whether carriage of multiple plasmids may result in a competitive cost to these *E. coli* strains that was not evident in pure cultures. At no time throughout the study was there a statistical difference in the *E. coli* populations grown on BHIA-T versus BHIA for each strain indicating the presence of a plasmid or chromosomal tetracycline resistance determinant throughout the study. Although the replicate isolates for strain 840 showed no loss of replicons throughout the culture period, all of the day36 isolates lost resistance to all antimicrobials except tetracycline. Thus, this suggested that harboring multiple plasmids was not sufficient in itself to ensure maintenance of the resistance phenotype. Since strain 840 did not transfer tetracycline resistance to any of the recipients tested, the tetracycline resistance determinants may be located on the bacterial chromosome or a nonconjugative plasmid. This does not rule out the possibility that other resistance genes may be located on mobile genetic elements. Lack of resistance gene maintenance or expression could occur by many mechanisms that were beyond the scope of this study.

Few isolates demonstrated replicon loss throughout the course of this project. Of 280 isolates characterized by PBRT, only three showed the loss of one replicon and only one of those displayed a phenotypic change in susceptibility. Although there were no differences in replicon profiles between the day0 strains and the day36 replicate isolates, there were differences in susceptibility and plasmid profiles. Acquisition of nalidixic acid and trimethoprim-sulfamethoxazole resistance occurred in one isolate, (831, d 36), without any apparent genotypic changes in plasmid or replicon profiles. This phenotypic change may have been due to chromosomal point mutations not being detectable by the methods used in this study. Two day36 isolates showed the loss of a single plasmid (1568 day36-4 and 155 day36-1). One of these two possessed only two plasmids initially (d 0) and the other possessed eight plasmids, suggesting that the number of plasmids a strain carried had little effect on the stability of the plasmid profiles. Two isolates contained plasmids that may have incurred deletions. The five 1568 day36 isolates displayed an additional plasmid band not observed in the corresponding wild-type strain as well as other changes that could have represented spontaneous genetic recombination. Since these cultures were pure cultures, an exogenous source (e.g., conjugation or transduction) for plasmid acquisition was not present.

Conclusion

Overall, these studies showed that there was no disparate plasmid loss among the strains studied regardless of the number of plasmids possessed. This suggested that there was little fitness cost to maintenance of multiple plasmids of various sizes by the strains under the conditions of this study. Strategies, other than merely reducing antimicrobial usage, are needed to reduce the increasing incidence of MDR bacteria

in the environment. Studies are currently underway to determine the fitness of these isolates in mixed-swine fecal cultures. This will also allow the opportunity to determine whether plasmid persistence in mixed culture is similar to that in pure culture.

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Disclosure Statement

No competing financial interests exist.

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