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Expression of a Cloned *Staphylococcus aureus* α-Hemolysin Determinant in *Bacillus subtilis* and *Staphylococcus aureus*

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A DNA sequence encoding *Staphylococcus aureus* α-hemolysin, which had been previously cloned and mapped in *Escherichia coli* K-12, was introduced into *Bacillus subtilis* BD170 and several strains of *S. aureus* by using plasmid vectors, some of which could replicate in all three organisms. The determinant was cloned on a 3.3-kilobase pair DNA fragment into *B. subtilis* by using the vector plasmid pXZ105 to form the hybrid plasmid pXZ111. *B. subtilis* cells harboring pXZ111 produced large zones of α-hemolysis after 18 h of growth at 37°C on rabbit blood agar plates, and α-hemolysin activity was detected in supernatants prepared from growing cultures of this strain. The α-hemolysin was apparently secreted across the *B. subtilis* cell envelope. Polypeptides of molecular weights 34,000 and 33,000 were precipitated with anti-α-hemolysin serum from lysates prepared from BD170 cells harboring pXZ111. A hybrid replicon which could replicate in both *E. coli* and *S. aureus* was constructed in *E. coli* by ligating a *Hin*dIII fragment encoding the replication functions and chloramphenicol resistance genes of *S. aureus* plasmid pCW59 to the pBR322 α-hemolysin hybrid plasmid pDU1150. The DNA of this plasmid, pDU1212, was prepared in *E. coli* and used to transform protoplasts prepared from a non-α-hemolytic, nonrestricting strain of *S. aureus* RN4220. Some of the transformants contained plasmids which had suffered extensive deletions. Some plasmids, however, were transformed intact into RN4220. Such plasmids were subsequently maintained in a stable manner. pDU1212 DNA was prepared from RN4220 and transformed into α-hemolytic *S. aureus* 8325-4 and two mutant derivatives defective in α-hemolysin synthesis. All three strains expressed α-hemolysin when harboring pDU1212.

The availability of a system for introducing cloned DNA fragments into different strains of *Staphylococcus aureus* would greatly simplify the methodology involved in constructing strains carrying specific mutations in single potential virulence determinants. Such strains would be extremely useful in identifying the role of individual determinants in the pathogenicity of *S. aureus* infections. Systems have already been developed for transforming DNA into some strains of *S. aureus* (9, 22, 24), and several plasmids have been identified which may be suitable for use as cloning vectors (11, 27). To date, few attempts have been made to reintroduce cloned *S. aureus* DNA fragments back into *S. aureus* strains either by using plasmid vectors or by homologous recombination after transformation with linear DNA. One approach to the problem would be to clone individual *S. aureus* virulence determinants which encode for extracellular proteins into *Escherichia coli* K-12 and carry out preliminary characterization of the determinants in this organism. Once the determinant has been mapped it could be reintroduced into *S. aureus* either on an *S. aureus* plasmid vector or on a composite plasmid "shuttle vector" which can replicate in either *E. coli* or *S. aureus*.

In an accompanying paper, the cloning and expression of the α-hemolysin determinant of *S. aureus* strain Wood 46 in *E. coli* K-12 was described (15). The α-hemolysin is a major *S. aureus* extracellular virulence factor which has toxic activity against a range of eucaryotic cells (12, 23). In this report, we describe our attempts to reintroduce the cloned α-hemolysin determinant back into different strains of *S. aureus*. The determinant was also introduced into *Bacillus subtlis* to form a strain which may act as a useful source of the α-hemolysin for purification procedures.

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TABLE 1. Bacterial strains used

Strain	Genotype	Source or reference
S. aureus		
8325-4		19
ISP2	8325 *nov-142*, *pig-131*, *hla*⁻ *-156*	22
ISP546	8325-4 *hla*⁻ *316*::Tn*551* *pig-131*	21
RN4220	*Hla*⁻, derivative of 8325-4	M. O'Reilly and R. P. Novick
B. subtilis		
BD170	*trp thr*	D. Ellis
E. coli K-12		
C600	*lac thr leu thi tonA rspR rspM*	24

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriological media. The bacterial strains and plasmids employed in this study are listed in Tables 1 and 2, respectively. *S. aureus* strains were propagated in tryptic soy broth (BBL Microbiology Systems, United Kingdom) or on tryptic soy agar plates. *B. subtilis* BD170 was propagated on L agar plates or in L broth.

Isolation of plasmid DNA. Plasmid DNA was prepared from *E. coli* (5), *B. subtilis* (3), and *S. aureus* (20) strains by cleared lysate formation. The DNA was purified from cleared lysates by cesium chloride-ethidium bromide centrifugation (5).

Transformation. Plasmid DNA was transformed into *E. coli* by the method of Cohen et al. (6). *B. subtilis* transformation was achieved with competent cells (7). Plasmid DNA was transformed into *S. aureus* protoplasts by the method of Chang and Cohen (4) with the modification that lysostaphin (Sigma London Chemical Co. Ltd., United Kingdom) at a final concentration of 10 μg/ml was used to generate protoplasts.

Enzymes. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) or from Boehringer Mannheim (Mannheim, West Germany) and were used according to the instructions of the manufacturer.

Labeling of whole cells, immunoprecipitation procedures, sodium dodecyl sulfate-polyacrylamide gel electrophoresis conditions, and autoradiography. Whole cells were prepared for radioactive labeling by inoculating 1 ml of an overnight culture into 100 ml of L broth, and the culture was grown with shaking at 37°C until the cells were at a density of between 2×10^8 to 4×10^8 cells per ml. Cells were harvested from 20 ml of the culture by centrifuging at 10,000 × *g* for 10 min; the pelleted cells were suspended in 20 ml of labeling medium consisting of 1× spiz salts (7), 1% (wt/vol) methionine assay medium (Difco Laboratories, United Kingdom), 0.5% (wt/vol) glucose, 50 μg of threonine per ml, 50 μg of tryptophan per ml, and 0.02% (wt/vol) $MgSO_4$ and were labeled for 1 h at 37°C with 150 μCi of [^{35}S]methionine (>100 Ci/mM; Amersham Radiochemical Centre, Amersham, United Kingdom). The labeled cells were harvested by centrifugation at 10,000 × *g*, suspended in 1 ml of lysozyme buffer containing 25% (wt/vol) sucrose, 30 mM Tris-hydrochloride, pH 8.0, 50 mM EDTA, 50 mM NaCl, and 1 mg of lysozyme per ml (Sigma) and incubated for 30 min at 37°C. The cells were then broken by sonication. Unbroken whole cells and cell debris were removed by centrifuging at 10,000 × *g* for 15 min, and the supernatant was retained. Polypeptides which reacted with anti-α-hemolysin sera were precipitated with fixed *S. aureus* Cowan 1 cells according to the method of Kessler (16). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were carried out as described previously (8).

Detection and measurement of α-hemolysin activity. Hemolytic activity was detected by streaking or tooth pick stabbing bacterial colonies onto L agar or tryptic soy agar plates containing 5% (vol/vol) washed rabbit erythrocytes. The levels of α-hemolysin production were measured by hemolytic titration. *S. aureus* strains were grown for 18 h at 37°C in 50 ml of Bernheimers broth (1) in baffled 250-ml flasks with shaking at 150 rpm. The supernatant was concentrated by precipitation with 90% ammonium sulfate. The precipitate was harvested and dissolved in 1 ml of

TABLE 2. Plasmids Used

Plasmid	Markers[a]	Comments	Source or reference
pDU1148	Cm^r Hla$^+$	pACYC184 carrying a 7.5-kbp *Hin*dIII fragment encoding α-toxin	15
pDU1150	Ap^r Hla$^+$	pBR322 carrying a 3.0-kbp *Hin*dIII-*Eco*RI fragment encoding α-toxin	15
pXZ111	Km^r Cm^r	pUB110 carrying a 1,031-base pair *Msp*I-*Mbo*I fragment encoding Cm^r derived from pC194	This study
pXZ105	Km^r Hla$^+$	pXZ111 carrying a 3.0-kbp fragment of pDU1148 encoding α-toxin	This study
pDU1212	Cm^r Ap^r Hla$^+$	pDU1150 linked to a 3.0-kbp fragment of pCW59 encoding Cm^r	This study
pCW59	Cm^r Tc^r		27
pUB110	Km^r		13

[a] Abbreviations: Hla$^+$, hemolysin production; Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

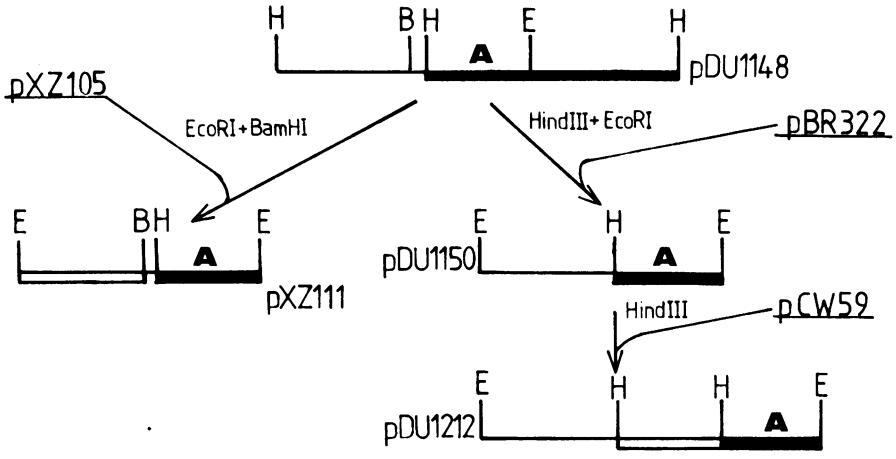

FIG. 1. Scheme for cloning α-hemolysin into *B. subtilis* and *S. aureus*. The thick dark line represents cloned *S. aureus* DNA, the open box represents pBR327 DNA for plasmid pXZ105 and pCW59 DNA for plasmid pDU1212. The thin line represents the other cloning vectors (see text). The restriction enzyme sites are represented by: H, *Hin*dIII: B, *Bam*HI; E, *Eco*RI. The letter A designates the α-hemolysin gene.

phosphate-buffered saline and dialyzed against 1 liter of phosphate-buffered saline for 18 h with one change. The α-hemolysin was then assayed (15).

Containment conditions. These experiments were conducted in accordance with the National Institutes of Health guideline for work carried out in Dublin and according to GMAG regulations for work carried out in Beckenham.

RESULTS

Plasmid vectors suitable for propagating cloned DNA in *E. coli* K-12, *B. subtilis*, and *S. aureus*. Plasmid pDU1148 contains a 7.6-kilobase pair (Kbp) fragment of *S. aureus* DNA encoding the α-hemolysin determinant cloned into the *Hin*dIII site of vector plasmid pACYC184 (15). Since *E. coli* plasmids such as pACYC184 do not replicate or express their drug resistance markers in gram-positive bacteria, it was necessary to link the α-hemolysin genes to plasmids that could replicate in gram-positive organisms before transfer into *B. subtilis* or *S. aureus*. Previous attempts by us to transform religated DNA directly into *S. aureus* had led to the formation of large deletions in the plasmids detected in transformants (M. O'Reilly and T. Foster, unpublished data). Therefore, two approaches were taken for cloning the α-hemolysin into gram-positive organisms. In the case of *B. subtilis*, DNA fragments encoding α-hemolysin production were ligated to the vector pXZ105 and the DNA was used directly to transform competent *B. subtilis* cells. For introduction into *S. aureus*, an *S. aureus* vector plasmid pCW59 was ligated to pDU1150 to form a shuttle vector capable of replicating in both *E. coli* and *S. aureus*. The pCW59-pDU1150 hybrid plasmid DNA was purified from *E. coli* cells and used to transform *S. aureus* protoplasts. Both approaches yield plasmids which should be capable of replicating in *B. subtilis* and *S. aureus*. The construction of the shuttle vectors was facilitated by the fact that the chloramphenicol and erythromycin resistance markers of gram-positive plasmids were expressed at detectable levels in *E. coli* (14; unpublished data).

Cloning of the α-hemolysin determinant in *B. subtilis*. The α-hemolysin determinant is encoded entirely within a 3.3-Kbp *Bam*HI-*Eco*RI fragment located on the *E. coli* plasmid pDU1148 (Fig. 1) (15). To ascertain whether this *Bam*HI-*Eco*RI fragment encoded sufficient information to express the α-hemolysin in a gram-positive organism, it was cloned into the *B. subtilis* host strain BD170 (Table 1). pDU1148 DNA was cleaved with *Bam*HI and *Eco*RI, and the linear fragments were ligated to *B. subtilis* vector plasmid pXZ105 DNA, which had been cleaved with the same enzymes. Transformants were selected directly by plating onto rabbit blood agar plates containing 5 μg of kanamycin per ml. After growth for 18 h at 37°C, large zones of hemolysis (ca. 5 mm in diameter) were detected around a number of the colonies. Some of the hemolytic colonies were propagated further on blood plates, and they remained kanamycin resistant and continued to synthesize hemolysin. Plasmid DNA was purified from several of these hemolytic transformants, and all were found to contain the 3.3-Kbp *Eco*RI-*Bam*HI fragment linked to the pXZ105 *Eco*RI-*Bam*HI fragment encoding the kanamycin resistance gene. Thus, in these recombinant plasmids, the 1.8-kilobase pair *Eco*RI-*Bam*HI fragment of pXZ105 encoding chloramphenicol resistance was replaced by the 3.3-kilobase *Eco*RI-*Bam*HI fragment of

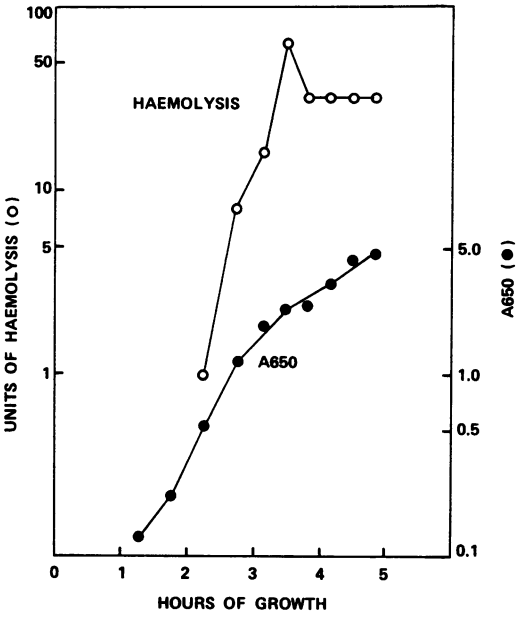

FIG. 2. Levels of α-hemolytic activity in supernatants of a growing culture of *B. subtilis* BD170 harboring pXZ111. BD170(pXZ111) and BD170(pUB110) were grown overnight in L broth containing 5 μg of kanamycin per ml at 37°C. The cultures were diluted into fresh L broth with kanamycin and were grown with aeration at 37°C. The hemolytic activity in the supernatant was determined after removal of whole cells by centrifugation at 10,000 × *g* at 4°C for 10 min. Hemolysis was measured by lysis of rabbit red blood cells in a 1:2 dilution series (see the text).

pDU1148, rendering the cells chloramphenicol susceptible. One of these plasmids was named pXZ111 and was used for further studies.

Expression of α-hemolysin in *B. subtilis*. Unlike the case in *E. coli* K-12 (15), the α-hemolysin appeared to be expressed extracellularly in *B. subtilis* as zones of hemolysis appeared after 18 h of growth on rabbit blood agar plates. To examine this expression in more detail, samples of supernatant prepared from growing cultures of *B. subtilis* BD170 harboring pXZ111 were assayed at different times throughout the growth cycle for hemolytic activity (Fig. 2). α-Hemolytic activity was detected continuously throughout the growth cycle in the culture supernatant. The activity reached a peak in the late-logarithmic phase of growth followed by a marked decline upon entering stationary phase. This decline could be due in part to the accumulation of *B. subtilis*-specified proteases in the culture supernatants.

The hemolysin synthesized by *B. subtilis* BD170 harboring pXZ111 was precipitated with specific anti-α-toxin serum. Polypeptides were labeled with [^{35}S]methionine, and the labeled cells were lysed as described above. Fixed *S. aureus* Cowan 1 cells possessing protein A were used to precipitate labeled polypeptides bound to anti-α-hemolysin serum from the cell lysate. An autoradiogram of the samples separated on a sodium dodecyl sulfate-polyacrylamide gel is shown in Fig. 3. A major band of molecular weight 34,000 and a minor band of molecular weight 33,000 were detected. This is close to the generally accepted value of the molecular weight of the α-hemolysin purified from *S. aureus* (18, 26). These bands were absent if precipitation was carried out with *B. subtilis* BD170 cells harboring pXZ105 or preimmune serum.

Expression of the cloned α-hemolysin determinant in *S. aureus*. A shuttle vector plasmid

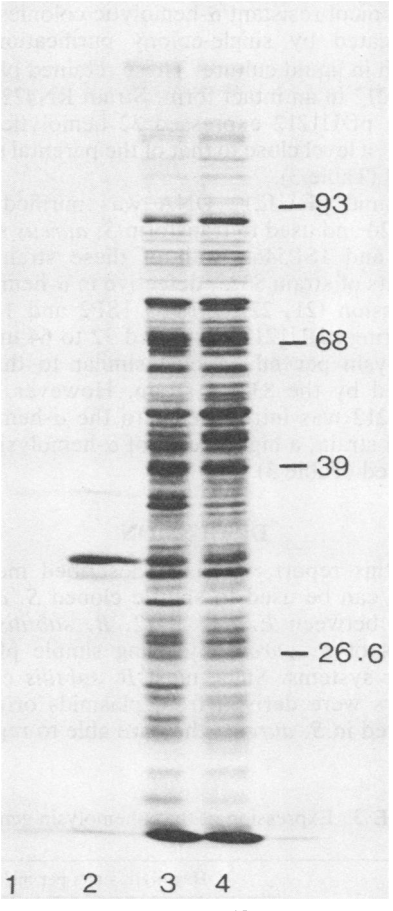

FIG. 3. Autoradiograph of [^{35}S]methionine-labeled extracts of *B. subtilis* BD170 and BD170(pXZ111) separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Tracks 1, *B. subtilis* BD170 immunoprecipitate; track 2, *B. subtilis* BD170(pXZ111) immunoprecipitate; track 3, *B. subtilis* BD170 total cell lysate; track 4, *B. subtilis* BD170(pXZ111) total cell lysate. Molecular weights are indicated (×10^6) on the right-hand side.

capable of replicating in *E. coli* and *S. aureus* was used to transfer the cloned α-hemolysin into *S. aureus*. The vector was formed in *E. coli* by linking *Hin*dIII-cleaved pDU1150 to the *Hin*dIII fragment of plasmid pCW59 encoding chloramphenicol resistance (Fig. 1). This construction was facilitated by the expression of the pCW59 chloramphenicol resistance in *E. coli*. The hybrid plasmid named pDU1212 was used to transform a hemolysin-defective *S. aureus* strain (RN4220). Most of the chloramphenicol-resistant transformants were hemolytic when plated onto rabbit blood agar. Some were nonhemolytic and were found to carry plasmids with extensive deletions. Colonies with small zones of hemolysis were composed of a mixture of cells, some carrying the hybrid plasmid intact and others carrying deleted plasmids. Several chloramphenicol-resistant α-hemolytic colonies were propagated by single-colony purification and growth in liquid culture. These retained plasmid pDU1212 in an intact form. Strain RN4220 harboring pDU1212 expressed 32 hemolytic units per ml, a level close to that of the parental strain, 8325-4 (Table 3).

Plasmid pDU1212 DNA was purified from RN4220 and used to transform *S. aureus* strains 1SP2 and 1SP546. Both of these strains are mutants of strain 8325, defective in α-hemolysin expression (21, 22). Strains 1SP2 and 1SP546 harboring pDU1212 expressed 32 to 64 units of hemolysin per ml, a level similar to that expressed by the 8325-4 strain. However, when pDU1212 was introduced into the α-hemolytic 8325-4 strain, a higher level of α-hemolysin was detected (Table 3).

DISCUSSION

In this report, we have described methods which can be used to shuttle cloned *S. aureus* genes between *E. coli* K-12, *B. subtilis*, and strains of *S. aureus* by using simple plasmid vector systems. Since most *B. subtilis* cloning vectors were derived from plasmids originally detected in *S. aureus*, they are able to replicate in both of these gram-positive organisms (11). Our findings confirm the observation that many gram-positive genes, including antibiotic resistance determinants, can be expressed in *E. coli*, although the pBR322 tetracycline and ampicillin resistances were not expressed in *S. aureus*. The α-hemolysin determinant of *S. aureus* Wood 46 was chosen for the study because it had already been cloned and mapped in *E. coli* K-12 (15).

Previous attempts to clone genes directly into *S. aureus* strains had been frustrated by the high frequency at which extensive deletions were found in any hybrid molecules which were detected in *S. aureus* after transformation with ligated DNA (M. O'Reilly and T. Foster, unpublished data). Cloning and physical mapping of the α-hemolysin determinant was simplified by carrying out all recombinant DNA manipulations in *E. coli* K-12 or *B. subtilis*. The frequency of introducing a cloned DNA sequence back into *S. aureus* in an intact form was increased greatly by using small, well-characterized DNA fragments and an *S. aureus* recipient that could be efficiently transformed with DNA. Eventually it may be useful to use nonreplicating DNA fragments to transform *S. aureus* so that mutated virulence genes can be reintroduced directly into the *S. aureus* genome by homologous recombination systems. Such an approach would lead to the substitution of the wild-type gene by the mutagenized gene as a single copy on the chromosome. This may be important as genes present in cells on multiple copy plasmids are often expressed at higher than normal levels (24, 25). It is interesting to note that the α-hemolysin-defective mutants of strain 8325 expressed α-hemolysin at the same level as wild-type α-hemolytic 8325-4 cells carrying a single chromosomal copy of the gene. This might indicate that the Wood 46 α-hemolysin gene is subject to regulatory signals characteristic of strain 8325-4.

Purification of the α-hemolytic protein from *S. aureus* culture supernatants has been hampered in the past by the instability of α-hemolysin expression (10, 17) and by low-level contamination by one or more of the other hemolytic agents expressed by *S. aureus* (12). Such contamination has confused biochemical studies on the mechanism of α-hemolysin action. Virtually nothing is known about how *S. aureus* cells regulate the expression of extracellular proteins. By introducing the α-hemolysin determinant into *B. subtilis*, we were able to detect active α-hemolysin in culture supernatants. The α-hemolysin was apparently secreted by the cells and expressed throughout the growth phase of the culture. Since *B. subtilis* does not express any compound with significant hemolytic activity, *B. subtilis* strains harboring the α-hemolysin deter-

TABLE 3. Expression of the α-hemolysin gene in *S. aureus*

Strain	Hemolytic units per ml[a]	
	No plasmid	With pDU1212
RN4220	<2	32
ISP546	<2	64
ISP2	<2	32
8325-4	64	768

[a] Hemolytic units per milliliter of concentrated culture supernatant prepared as described in the text. The numbers are mean values of two independent assays.

minant cloned on a multiple-copy plasmid could act as a useful source from which to purify the α-hemolysin for biochemical studies. The α-hemolysin can be used as a immunoprophylactic agent to give some protection against gangrenous mastitis (1) and as a diagnostic reagent for detecting anti-α-hemolysin antibodies in the sera of patients suffering from *S. aureus* infections.

Immunoprecipitation experiments carried out with *B. subtilis* BD170 harboring pXZ111 suggest that the α-hemolysin may exist as two distinct polypeptides of molecular weights 34,000 and 33,000. As yet we do not know whether these two forms represent different processed forms of the same polypeptide with and without a secretion signal sequence, although we are currently investigating this possibility.

We have shown that it is possible to clone and map the determinants for extracellular proteins of *S. aureus* in *E. coli* K-12 and introduce these cloned genes into *B. subtilis* or back into *S. aureus*. We intend to use this approach to characterize other *S. aureus* determinants, including the β-hemolysin and coagulase, and to use the cloned genes as a means to mutagenize genes in situ on the *S. aureus* chromosome.

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