Properties and Comparative Starch-gel Electrophoresis of Megacins from Several *Bacillus megaterium* Strains

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SUMMARY

The products of several megacinogenic strains of *Bacillus megaterium* were analysed by starch-gel electrophoresis. All were mixtures of inhibitors, among which megacins A, C and Cx could be distinguished. Several non-inducible megacins were produced in liquid medium and they seemed to be a heterogeneous group which may correspond to 'megacin B'. The effects of heat and proteolytic enzymes on megacins were investigated and the molecular weights of several megacins were estimated by chromatography using Sephadex G 200. The molecular weight of crude megacin C was about 153 000. Strains producing megacins C and Cx could be 'cured' by growth at 43 °C. Megacins C and Cx were released from the surface of meg⁺ organisms by partial digestion of the wall with trypsin or lysozyme.

INTRODUCTION

Megacins, bacteriocins produced by *Bacillus megaterium*, appear to be a rather heterogeneous group. First discovered by Ivanovics & Alföldi (1954), they were provisionally classified into three groups by Holland & Roberts (1964) using the criteria of production characteristics, spectrum of activity and mode of action. Megacin A was defined as being inducible, having a very wide spectrum of activity, and acting on the cytoplasmic membrane of sensitive organisms to cause lysis. Megacin A is now known to be a phospholipase A₂ (Ozaki *et al.* 1966; Ochi *et al.* 1971). Megacin B was characterized by a narrower spectrum of activity, being non-inducible and by being produced only on solid medium. Megacin C had a very narrow spectrum of activity and was produced by uninduced cultures in the late exponential phase. The megacin produced by *B. megaterium* 337 (Durner & Mach, 1966) was provisionally called megacin Cx, as the characteristics of its production were similar to megacin C. This megacin has been purified by Durner (1970*a*) and its mode of action differs from that of crude megacin C (Durner, 1970*b*; Holland, 1965).

Preliminary experiments showed that megacins could easily be distinguished by starch-gel electrophoresis. It is a very gentle procedure, which is necessary as many megacins are unstable. Since starch-gel is non-toxic, the position of megacins can be shown by inhibition zones in an overlying sensitive strain, which is a method far more sensitive and specific than using stains.

METHODS

Organisms. *Bacillus megaterium* strains 216 (megA⁺C⁺), 216A⁻ (megA⁻C⁺) and 337 (megCx⁺) were obtained from Professor G. Ivanovics, Institute of Microbiology, University Medical School, Szeged, Hungary. *Bacillus megaterium* C4A⁻ (megA⁻C⁺) was obtained from Dr I. B. Holland, Department of Genetics, Leicester, England. Strain 216(31) is a

non-inducible mutant isolated from *B. megaterium* 216. Strains C4- and 337- are megC⁻ and megCx⁻ respectively. The departmental strains B1, B2 and B4 are non-inducible megacin producers. Other strains were obtained from soil samples according to the method of Holland & Roberts (1964). Strain 192 is an inducible meg⁺ strain and 34 is a non-inducible meg⁺ strain. *Bacillus megaterium* 162u is a streptomycin-resistant indicator strain which is far more sensitive to megacins than all other strains tested.

Media. Oxoid blood agar base no. 2 (4%, w/v; NA) was used for routine subculture of strains at 37 °C. Peptone water (PW) was the routine liquid medium containing 1% (w/v) each of bacto-peptone and bacto-tryptone, and 0.05% (w/v) NaCl. Soft agar used for overlaying plates contained Difco nutrient broth 1.6% (w/v), NaCl 0.5% (w/v) and bacto agar 0.7% (w/v).

Peptone glucose broth (PGB) (Holland, 1961) was used for megacin production. The PRE medium of Cundliffe (1968) containing 10% (w/v) sucrose and 0.1% (w/v) peptone was used for cultures before their conversion to protoplasts and for their subsequent incubation as protoplasts.

Buffers. Discontinuous buffers for starch-gel electrophoresis were tris-citrate buffer, pH 8.60 to 8.65 (gel), and borate buffer, pH 8.60 to 8.65 (bridge solution) (Poulick, 1957). Phosphate buffer was equal volumes of 0.1 M-KH₂PO₄ and 0.1 M-K₂HPO₄, pH adjusted to 7.20 with NaOH. Barbitone buffer was prepared from stock solution A, 0.2 M-sodium barbitone (sodium diethyl barbiturate) and stock solution B, 0.2 M-HCl; (50 ml A + 9 ml B + 14 ml water to give pH 8.40).

Chemicals. Lysozyme from hen egg, A grade, was purchased from Calbiochem Ltd, London, W1H 1AS; hydrolysed starch from Connaught Medical Research Laboratories, Toronto, Canada; streptomycin sulphate from Glaxo Laboratories Ltd, Greenford, Middlesex Blue Dextran and Sephadex (G 200 and G 25) from Pharmacia (G.B.) Ltd, London, W. 5. Pure α -chymotrypsin ex bovine pancreas, pure human γ -globulins Cohn fraction II, pure myoglobin from sperm whale, papain (twice crystallized), pure pepsin (from hog pancreas and tris puriss A.R. were obtained from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire. Sigma Chemical Co., London supplied bovine serum albumin (fraction V and pure preparations), α -chymotrypsinogen A type 2, crystalline mitomycin C, ovalbumin A.R. Grade V (pure) and trypsin type III, pure.

Megacin production. Overnight PW cultures of the producer strain were inoculated into PGB so that they were diluted 25-fold and incubated on a shaker in a 35 °C water bath. Strain 216 was induced to produce megacin A by adding mitomycin C (0.2 μ g/ml) to a culture in the early exponential phase of growth. On re-incubation, the megacin was released by lysis approximately 2 h later.

Megacins C and Cx were harvested when the culture of the producing organism was in late exponential phase and all other megacins, including those produced by uninduced megA⁺ strains, were harvested when the cultures were in the stationary phase. The bacteria were centrifuged at 10000 g for 30 min at 5 °C and bovine serum albumin (1 mg/ml) was dissolved in the supernatant liquid (Mitusi & Mizuno, 1969). The preparation was dialysed overnight at 5 °C against 1.0 mM-phosphate buffer, pH 7.20. The megacins were then shaken with chloroform to reduce contamination and freeze-dried.

The freeze-dried megacins were prepared for use by making a saturated solution in sterile distilled water. Occasionally freshly prepared crude supernatant liquids from cultures were used instead of freeze-dried preparations.

Megacin assay. A spot method similar to that of Ivanovics & Alföldi (1955) was used. Plates of NA containing streptomycin (10 μ g/ml) were overlaid with 4 ml of soft agar

containing 0.1 ml of an overnight PW culture of the indicator strain 162u. The megacin preparations were diluted in saline and spotted on to the seeded agar surface using a standard loop. The reciprocal of the highest dilution giving detectable clearing of the indicator was taken as the megacin titre in arbitrary units/ml. Induced preparations contained 1×10^4 to 1×10^8 arbitrary units/mg protein. Freshly prepared uninduced megacins had titres from 0.1 to 10 arbitrary units/mg protein.

Lysozyme treatment and preparation of protoplasts. The method of Cundliffe (1968) was used with minor modifications. Organisms in the exponential phase were treated with lysozyme (500 μ g/ml) in PRE medium at 37 °C until they were seen by phase-contrast microscopy to have been converted into protoplasts. The protoplasts were diluted 20-fold with fresh medium and incubated at 37 °C for about 18 h while gently shaking.

When the supernatant liquids after lysozyme treatment were to be tested for megacin activity, the bacteria were washed thoroughly in warm PRE medium before lysozyme treatment. After protoplast formation the preparation was centrifuged and the supernatant liquid removed.

Starch-gel electrophoresis. The method was essentially that of Smithies (1955), with the discontinuous buffer system of Poulick (1957). During the experiments air was excluded from the gel by a 'terphane' polyester film (BCL Plastic Films Division, British Cellophane Ltd, Regal House, Twickenham, Middlesex). Samples were inserted into the gel on Whatman no. 3 mm filter-paper strips that had been blotted. The samples were placed near the cathode and a potential of 1 V/mm gel was used. Megacin from induced 216 was usually included in each run to provide an internal standard. Runs were continued for 5 to 6 h until the buffer line had almost travelled to the anode, about 105 mm from the cathode. The gel was then sliced into horizontal layers which were placed in flat dishes and each overlayered with NA seeded usually with 1.5 ml of overnight PW culture of an indicator strain per 15 to 20 ml of soft agar held at 55 °C. After overnight incubation at 37 °C the slices were examined for inhibition zones by placing them against a dark background with oblique lighting. The positions of the zones were carefully measured and drawn on accurate full-scale plans. Four slices from each run were overlayered with different indicator strains or were used as replicates.

Heat-treatment of megacins. Megacin solutions were held for 1 h in a water bath at 60 °C. A control solution of the same megacin was held for 1 h at 37 °C.

Treatment of megacins with proteolytic enzymes. Trypsin and chymotrypsin (each 1 mg/ml) were dissolved in 0.04 M-barbitol buffer, pH 8.0. Pepsin (1 mg/ml) was dissolved in 0.1 M-glycylglycine buffer, pH 2.8 to 2.9. Undiluted papain suspension (0.3 ml) was mixed with 5 ml of 0.1 M-sodium acetate, pH 4.5, containing 0.01 M-cysteine and 0.01 M-EDTA (Kemmel & Smith, 1954). Equal volumes of megacin and each enzyme solution were mixed and left at 37 °C for 1 h. As a control, buffer alone was added to the megacin.

Molecular weight estimation. A Pharmacia column (internal diameter 25 mm) was packed with Sephadex G 200 to a depth of approximately 40 cm. A thin layer of Sephadex G 25 was added to the top of the column to prevent damage to the bed during the addition of samples. Phosphate buffer, 0.01 M, pH 7.2, was used at 2 °C and a fraction collector and Uvicord (LKB-Produkter AB, Sweden) were connected. At the start of every run a 2% (w/v) Blue Dextran solution (1 to 2 ml) was added to estimate the void volume (V_0). Each sample was made of high density by saturating with sucrose. The standard curve was constructed with γ -globulin, bovine serum albumin, ovalbumin, α -chymotrypsinogen and myoglobin (Andrews, 1964). During standardization of the column, 10 to 15 mg of each standard was dissolved in the Blue Dextran solution and up to three standards were used

Fig. 1

Fig. 2

Fig. 1. Starch-gel electrophoresis of megacins. The producing stains were: induced 192 (1), 192 (2), C4A⁻ (3), 216 (31) (4), 216 (5) and induced 216 (6). The positions of the megacins (freshly are shown by the prepared zones of inhibition of the indicator strain 162u. The bar represents 1 cm.

A = megacin A; C = megacin C.

Fig. 2. Starch-gel electrophoresis of megacins. The plan shows the megacin from C4A⁻ (1) and the effect of treatment with heat (2), chymotrypsin (3) and trypsin (4) on megacin from induced 216, compared with the untreated control preparation (5). The indicator strain is 162u. The bar represents 1 cm.

A = megacin A; C = megacin C.

per run. Megacin preparations were usually in the form of a freeze-dried powder and as much as possible was dissolved in the 1 to 2 ml of the Blue Dextran solution. The fractions were collected and assayed for megacin activity. The results, in arbitrary units/ml, were plotted on to the Uvicord graph and the elution of the peak of the activity determined (V_i) and hence the V_i/V_0 ratio. From the standard curve, of V_i/V_0 against log molecular weight, the molecular weight of the sample was calculated.

RESULTS

Preliminary electrophoretic studies

Freshly prepared megacins gave several distinct inhibition zones and one of these was immobile (Fig. 1). In older or freeze-dried preparations, only the major components of each preparation were visible. The inhibition zones were assumed to be caused by megacins. They were not found in preparations from meg⁻ strains. Zones caused by bacteriophage would be immobile and increase in size during incubation; this was never seen.

The most mobile zone (mobility approximately 15 mm/h) probably corresponds to megacin A. Little was present in preparations from 216(31) or uninduced 192 or 216, yet there was a huge increase in this component in induced 192 or 216 cultures. The major component produced by C4A⁻ is assumed to be megacin C. If so, both 216 and 216(31) produce small amounts of megacin C because a zone of identical mobility is seen when their

Table 1. *The composition of different megacin preparations shown by starch-gel electrophoresis*

The positions of megacins were recognized by the zones of inhibition of the indicator organism (*Bacillus megaterium* 162u).

Meg ⁺ strains	Components present							
	Mobility							
	(expressed as averaged fraction of mobility of megacin A)							
	Immobile	0.05	Megacin		0.61	0.74	0.83	Megacin A 1.00
Cx 0.10			C 0.39					
192 (induced)	+			Unresolved inhibitors				+
192	+	—	—	—	—	—	—	+
216 (induced)	+	—	—	+	+	+	+	+
216	+	—	—	+	—	—	—	+
216(31)	+	—	—	+	—	—	—	+
'216A ⁻ '	+	—	—	—	—	—	—	+
C4A ⁻	+	—	—	+	—	—	—	+
337	—	—	+	—	—	—	—	—
B1	—	+	—	—	—	—	—	—
B2	—	—	—	—	—	—	—	+
34	+	—	—	—	—	—	—	—

products are electrophoresed (Fig. 1). Strain '216A⁻', described as being a mutant producing only megacin C and not megacin A (G. Ivanovics, personal communication), appears in other experiments to be similar to 216(31), but a megacin C zone has never been demonstrated in its supernatant liquid. Strain C4A⁻ is apparently an uninducible mutant like 216(31), but produces a large proportion of megacin C and a very small proportion of megacin A and the immobile component. The position of the megacin C inhibition zone was confirmed, using C4A⁻ (megC⁺) and the negative mutant, C4⁻, as indicators. The zone was visible only when using strain C4⁻ as the indicator. The megacin C-producing strain, C4A⁻, is sensitive only to very high concentrations of megacin C. Megacin Cx, produced by strain 337, could be distinguished by a distinct zone which moved only a short distance from the cathode (Fig. 4). This was confirmed by using both strain 337 and the megC⁻ mutant, strain 337⁻, as indicators. A zone of inhibition was visible only when the negative mutant was used. Other megacins were much weaker, and some appeared immobile under the conditions of the experiment so that they could not be distinguished from each other. Generally only strain 162u, the most sensitive indicator strain, was inhibited by them. The preparation from induced 192 may contain other components but these cannot be resolved as the indicator strain is inhibited over the whole area of gel between the base-line and the very mobile component. Megacin from induced 216 contains additional weak components found in fresh preparations, presumably released by lysis (Table 1).

Megacin A

Both strains 192 and 216 were inducible and both produced a megacin with the same electrophoretic mobility. These two megacins differed, however, in their susceptibility to heat and proteolytic enzymes. Megacin from induced 192 was very heat-sensitive and was inactivated by trypsin treatment. Neither trypsin nor chymotrypsin had any observable effect on megacin from induced 216 (Fig. 2). However, trypsin treatment decreased the amount of clearing on plates of the indicator although the titre appeared the same. The

Fig. 3

Fig. 4

Fig. 3. Starch-gel electrophoresis of megacin from induced *Bacillus megaterium* 216. Organisms were harvested at 1½ h (1, 2), 2 h (3, 4) and 3 h (5, 6) after lysis. The earliest sample (1, 2) shows no activity. Preparations 2, 4 and 6 were heat-treated, and preparations 1, 3 and 5 are the untreated controls. The indicator is 162u. The bar represents 1 cm.

A = megacin A.

Fig. 4. Starch-gel electrophoresis of the products of lysozyme treatment of *Bacillus megaterium*. Lysozyme solution (1.5 mg/ml) (1); the supernatant liquid obtained by treating washed C4- (2) and C4A- (3) organisms with lysozyme in PRE medium; supernatant liquid from strain C4A- growing in PRE medium (4); and the supernatant liquid obtained by treating washed 337 organisms with lysozyme in PRE medium (5). The indicator is 162u. The bar represents 1 cm.

C = megacin C; Cx = megacin Cx; L = lysozyme.

molecular weights of the two megacins are different. That of the megacin from strain 192 was estimated to be about 68 000 whereas megacin from strain 216 has a molecular weight of 51 000 (Holland, 1961). However, megacin A preparations from different sources may have identical modes of action, although having slightly different physical properties (Nagy, Alföldi & Ivanovics, 1959).

The effect of heat treatment on induced megacin from strain 216 is shown in Fig. 2. The mobile component is apparently converted into a less mobile component on heating. There is some evidence from agar-gel electrophoresis (Seed, 1970) that the less mobile component is converted into the mobile component on prolonged incubation of the induced culture. Prolonged incubation of the lysed induced culture definitely increases the proportion of the mobile component converted to the less mobile component which occurs on heating (Fig. 3).

Because megacins C and Cx may be wall components (see below), the production of megacin A by protoplasts was investigated. Protoplasts of strain 192 produced both mobile and immobile components in amounts comparable to those in supernatant liquids from uninduced cultures. This demonstrates that megacin A production did not depend on the presence of a bacterial wall.

Table 2. Summary of the physical and chemical properties of megacins

	Source of megacins											
	Induced 192	Induced 192	Induced 216	216	216(31)	'216A ⁺	337	C4A ⁻	B1	B2	B4	34
Sensitivity to												
Heat	+	+	-	-	-	-	+	+	+	±	-	+
Trypsin	+	+	±	+	+	±	-	-	-	+	±	+
α-Chymotrypsin	+	+	-	+	+	+	±	-	-	+	±	+
Pepsin	±	-	±	-	+	+	+	±	-	+	±	+
Papein	±		±	±	+	+	±	-	±		±	+
Main megacin present	A	A	A				Cx	C	?			
Molecular weight	68000	51000 (Holland, 1961)	163000 (160000 Durner, 1970a)					153000	50000			
				</								

+ Sensitive; ± partially sensitive; - insensitive.

Megacin B

The megacins produced by many strains in the stationary phase of growth are assumed to be megacin B. The harvesting time is critical for detecting these megacins, which may be the reason why other workers have not obtained non-inducible megacins other than C and Cx in liquid medium. The molecular weight of one such megacin (from strain B1) was about 50000, which is comparable to that of megacin A. This group of megacins are heterogeneous and differ in their sensitivities to heat and proteolytic enzymes (Table 2).

Megacins C and Cx

These megacins are considered together as they have many properties in common, in spite of their different modes of action. Their distinct electrophoretic mobilities may be due to the differences in their molecular weights (Table 2).

Both strains C4A⁻ and 337 appeared to lose the meg⁺ character rather easily, and this loss was examined further by replica plating. High numbers of meg⁻ clones could always be isolated from meg⁺ cultures of both producer strains, and there was almost 100% conversion of meg⁺ cultures to meg⁻ when meg⁺ cultures were grown in PW overnight at 43 °C, then spread on NA and incubated and replicated on to an indicator at 37 °C. Conversion of meg⁺ to meg⁻ cultures by chemical agents has not yet been demonstrated. Meg⁻ clones have never shown any reversion to the meg⁺ state. Many mixed clones, containing meg⁺ and meg⁻ organisms, were isolated during the investigation.

Holland & Roberts (1964) have suggested that megacin C may be a wall component which is in equilibrium between bound and free states. Protoplasts, therefore, would not be expected to produce this megacin. This seems to be the case, as supernatant liquids from protoplasts of strain C4A⁻ contain all components except megacin C. One reason for this may be that, although the protoplasts were prepared from cultures in the exponential phase of growth, the lysozyme treatment may have shocked the organisms, thus preventing megacin C synthesis and release. The protoplasts were shown to be metabolically active, however, by their reduction of triphenyl tetrazolium chloride (Tengerdy, Nagy & Martin, 1967). Additional evidence that megacins C and Cx are wall-bound was given by electrophoresis of supernatant liquid obtained when washed meg⁺ bacteria were treated with lysozyme. An inhibition zone due to the lysozyme was visible, and also a specific zone due to either megacin C or Cx, depending on the producer strain used (Fig. 4). This was confirmed using the meg⁺ and the meg⁻ mutant strains as indicators. As also shown in Fig. 4, the meg⁺ organisms had already released megacin into the production medium before being converted into protoplasts. Thus the megacin released by lysozyme from the washed meg⁺ organisms may have only adsorbed to the walls and not formed there. A similar result was obtained if bacteria grown in PGB medium were used, although such organisms are not converted into protoplasts by lysozyme. An attempt was made to dissociate the megacin from the meg⁺ wall by treatment with 8 M-lithium chloride (Pooley, Porres-Juan & Shockman, 1970) but this was unsuccessful.

Megacins C and Cx were both trypsin-resistant, yet preliminary experiments of the 'trypsin-rescue' type (Reynolds & Reeves, 1963) showed that immediate treatment of organisms that had adsorbed an excess of megacin led to partial rescue. This suggests that the megacin may be removed by digestion of its adsorption site on sensitive bacteria.

When washed megC⁺ and megCx⁺ organisms (strains C4A⁻ and 337) were pretreated with trypsin and then washed again before the lysozyme treatment, electrophoresis of the lysozyme supernatant liquid showed a decrease in the amount of megacin released, com-

pared to the controls that were not treated with trypsin. Hence, most of the megacin released by digestion of meg⁺ walls is on the outer surface. There is no indication whether the remaining megacin is also on the outer surface or is on the inner surface, as would be the case if it were produced in the periplasmic space. When meg⁺ organisms were grown in both PRE and PGB and treated with lysozyme with or without trypsin pretreatment, the washed suspensions of organisms had differing inhibitory activities. Those grown in PGB were more inhibitory to the indicator strain than those grown in PRE, and trypsin pretreatment increased the inhibition. The difference between the two media probably appears because lysozyme only partially digests the PGB-grown wall, so that less megacin is removed. The effect of the trypsin pretreatment may be to expose deeper layers of the wall to the lysozyme. The increased inhibitory activity of the remaining suspension suggests that there may be more megacin towards the inner wall surface.

DISCUSSION

The present work shows that there are at least three genes concerned with megacin production in strain 216: megacin A production, inducibility and megacin C production. According to Ochi *et al.* (1970), another associated product is that of the specific megacin A inhibitor. Strain 216 possesses all of these characteristics. Electrophoretic analysis has shown that strain 216(31) is a non-inducible mutant, though still producing megacins A and C. Strain '216A⁻' is also a non-inducible mutant, producing megacin A but apparently not megacin C. The highly mobile megacin A component is shown by electrophoresis to be converted into a less mobile component on heating. The relationship between these two components is not yet understood. The megacin A inhibitor is reported to be reversible in action, trypsin-resistant and heat-sensitive (Ochi *et al.* 1970, 1971). The apparent conversion of the mobile into a less mobile component may be correlated in some way with the destruction of this megacin inhibitor. The weak megacins released in the stationary phase of growth of producer cultures may correspond to the group 'megacin B'. They are heterogeneous, although at least one has a molecular weight similar to that of megacin A. They may be released on autolysis as they are detected only at the end of the growth phase of meg⁺ cultures.

Megacins C and Cx are both released from the walls of meg⁺ organisms by lysozyme treatment and the trypsin-pretreatment experiment suggests that the megacins are on the outer wall surface. The site of production is not yet known but it must be inside the organism (although protoplasts do not produce them) or in the periplasmic space. Megacins must somehow find their way through the wall, which is surprising in view of their high molecular weights. Isolated walls of *Bacillus megaterium* KM exclude uncharged molecules with a molecular weight greater than 57000 (Gerhardt & Judge, 1964). However, there is extensive turnover of wall material in *B. megaterium* during the exponential phase (Chaloupka, Říhová & Křečková, 1964). Megacins C and Cx may be released into the culture fluid at this time as a result of such turnover.

The rapid conversion of meg⁺ to meg⁻ organisms which occurs at 43 °C suggests that possibly both the megC⁺ and megCx⁺ determinants may be on plasmids. It is not known, however, if such plasmids would be comparable to those in organisms such as *Escherichia coli*. Carlton & Helinski (1969) obtained results suggesting that most of the DNA in both meg⁺ and meg⁻ *Bacillus megaterium* strains is present as several covalently linked closed circles. They could not distinguish an additional DNA circle in meg⁺ strains.

Megacins are obviously a very diverse group of inhibitory substances, as shown both

by their molecular properties and their modes of action. Bradley (1967) has devised a classification system for bacteriocins which uses such characteristics as thermostability, molecular weight and sensitivity to proteolytic enzymes to distinguish bacteriocins derived from viruses or wall components. Megacins, however, cannot be fitted into this scheme. The activity spectra of megacins have already been used by the author to classify them by constructing a similarity matrix and performing a cluster analysis (Seed, 1970). This results in a grouping which is consistent with current information. An ideal classification scheme should also include all the known properties of megacins such as those discussed in this paper. At the present time this would give a more realistic picture of megacin classification than a scheme dependent on 'key' characters.

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