Isolation and Characterization of a Recombinant Plasmid Carrying a Functional Part of the *Bacillus subtilis* *spoIIA* Locus

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Plasmid pHM2 consists of a 3·3 kb insert of *Bacillus subtilis* DNA in the chimeric plasmid pHV33, and can replicate in *Escherichia coli* and *B. subtilis*. In *B. subtilis*, pHM2 complements the defects resulting from mutations *spo-42*, *spo-50*, *spo-69* and *sas-1* in the *spoIIA* locus. This complementation can occur in *recE4* strains where recombination of the plasmid with the chromosome is prevented, and the chromosome retains the mutant allele. Thus the plasmid carries a functional part of the *spoIIA* locus; it does not contain the complete locus as it cannot complement several other *spoIIA* mutations. It is likely that the locus is complex, containing at least two genes.

INTRODUCTION

Spore formation in *Bacillus subtilis* has been widely used as a model system for studying cellular differentiation. Some 35 genetic loci have been identified by mutations that block sporulation (Piggot & Coote, 1976). The study of cloned DNA should help us to understand how expression of these loci is controlled during sporulation. Three of the loci, *spoIIA*, *spoIIE* and *spoIIIA*, appear to be polygenic from their genetic maps (Piggot, 1973; Piggot & Coote, 1976). Consequently, it seems to be particularly useful to study these loci.

We report here the isolation and characterization of a plasmid carrying a functional part of the *spoIIA* locus. The plasmid was isolated from a clone bank of sheared *B. subtilis* DNA inserted by A·T tailing into the *Bam*HI site of the plasmid pHV33 (Rapoport *et al.*, 1979). Plasmid pHV33 is a chimera of the *Escherichia coli* plasmid pBR322 and the *Staphylococcus aureus* plasmid pC194, and can replicate in *E. coli* and *B. subtilis* (Ehrlich, 1978; Primrose & Ehrlich, 1981). The plasmid confers resistance to chloramphenicol in *B. subtilis*, and resistance to chloramphenicol, ampicillin, and tetracycline in *E. coli*; insertion of foreign DNA into the *Bam*HI site inactivates tetracycline resistance.

METHODS

Strains. The strains used are listed in Table 1. The *B. subtilis* strains were derivatives of the transformable strain 168. The Rec⁻ phenotype was checked before each experiment with Rec⁻ strains. *B. subtilis* *recE4* strains were tested for their inability to grow on nutrient agar containing 50 ng mitomycin C ml⁻¹. *Escherichia coli* *recA* strains were tested for their sensitivity to UV irradiation. In general, *B. subtilis* strains were maintained on nutrient agar, and *E. coli* strains on L-agar; appropriate antibiotics were included for strains harbouring plasmids: chloramphenicol, 5 µg ml⁻¹ for *B. subtilis*, 10 µg ml⁻¹ for *E. coli*; ampicillin, 25 µg ml⁻¹.

Media. L-agar contained, per litre: Difco tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 10 g; Difco agar, 16 g; adjusted to pH 7·0. Transformation medium (Bott & Wilson, 1968) was made up shortly before use by mixing 100 ml Bott & Wilson salts, 1 ml 50% (w/v) glucose, 0·6 ml 1 M-MgSO₄ and 5 ml Bott & Wilson amino acids. This was supplemented with 10 µg tryptophan ml⁻¹, and other amino acids at 100 µg ml⁻¹ where appropriate. Bott &

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Table 1. *Bacterial strains*

<i>B. subtilis</i> strain	Genotype	Origin/reference
168T ⁺	Prototroph	Piggot (1973)
BR151	<i>metB10 lys-3 trpC2</i>	R. S. Buxton
GSY908	<i>argC4 hisA1 recE4</i>	C. Anagnostopoulos
MY2010	<i>hisA pyrD1 rfm sas-1</i>	Yudkin & Turley (1980)
SL401	<i>spoIIA1 trpC2</i>	E1, Piggot (1973)
SL437	<i>spoIIA37 trpC2</i>	NG1.82 Piggot (1973)
SL442	<i>spoIIA42 trpC2</i>	NG6.13 Piggot (1973)
SL450	<i>spoIIA50 trpC2</i>	NG11.2, Piggot (1973)
SL976	<i>spoIIA42 trpC2 metB10</i>	DNA.SL442 × BR151→Lys ⁺
SL977	<i>sas-1 trpC2 metB10</i>	DNA.M2010 × BR151→Lys ⁺
SL978	<i>spoIIA1 trpC2 metB10</i>	DNA.SL401 × BR151→Lys ⁺
SL979	<i>spoIIA5 trpC2 metB10</i>	DNA.4SA (Ionesco & Schaeffer, 1968) × BR151→Lys ⁺
SL980	<i>spoIIA4 trpC2 metB10</i>	DNA.4Z (Ionesco & Schaeffer, 1968) × BR151→Lys ⁺
SL981	<i>spoIIA12 trpC2 metB10</i>	DNA.12U (Ionesco & Schaeffer, 1968) × BR151→Lys ⁺
SL983	<i>spoIIA26 trpC2 metB10</i>	DNA.26U (Ionesco & Schaeffer, 1968) × BR151→Lys ⁺
SL984	<i>spoIIA16 trpC2 metB10</i>	DNA.16U (Ionesco & Schaeffer, 1968) × BR151→Lys ⁺
SL986	<i>spoIIA42 metB10 recE4</i>	DNA.GSY908 × SL976→Trp ⁺
SL988	<i>sas-1 trpC2 recE4</i>	DNA.GSY908 × SL977→Trp ⁺
SL1013	<i>spoIIA69 lys-3 trpC2</i>	DNA.69.3 (Piggot & Taylor, 1977) × BR151→Met ⁺
SL1060	<i>spoIIA69 trpC2 recE4</i>	DNA.GSY908 × SL1013→Lys ⁺
SL1067	<i>spoIIA50 recE4</i>	DNA.GSY908 × SL450→Trp ⁺
SL3024	(pHM2) <i>spoIIA69 trpC2 recE4</i>	See text.
<i>E. coli</i> strain	Relevant genotype	Origin/reference
SL2004	(pHV33) <i>thi leu pro lac ton supE hsdR str</i>	HVC45(pHV33) Primrose & Ehrlich (1981)
SL2009	<i>hsdR supE recA56</i>	<i>recA56</i> introduced into a Q358 (Karn <i>et al.</i> , 1980) derivative by P1 <i>clm clr-100</i> transduction (Csonka & Clark, 1980)
SL3030	(pHM2) <i>hsdR supE recA56</i>	See text
RB342	(λ c1857S7) <i>lac(Am) trp(Am) sup</i>	R. S. Buxton

Wilson salts consisted of K₂HPO₄, 1.24% (w/v); KH₂PO₄, 0.76% (w/v); trisodium citrate, 0.1% (w/v); (NH₄)₂SO₄, 0.6% (w/v); adjusted to pH 6.7. Bott & Wilson amino acids contained valine, lysine, threonine, glycine, aspartic acid, methionine, histidine, tryptophan and arginine, each at 500 µg ml⁻¹. PAB-Cm was Penassay broth (Difco antibiotic medium no. 3) containing 5 µg chloramphenicol ml⁻¹. Other media have been described previously (Piggot, 1973).

Transformation. *Bacillus subtilis* was transformed by the method of Bott & Wilson (1968) and *E. coli* transformed by the method of Brown *et al.* (1979). DNA was used at 1 µg ml⁻¹ for transformation. Chloramphenicol-resistant (Cm^R) transformants of *B. subtilis* were selected on nutrient agar containing 5 µg chloramphenicol ml⁻¹. Ampicillin-resistant (Amp^R) transformants of *E. coli* were selected on L-agar containing 25 µg ampicillin ml⁻¹. Sporulating (Spo⁺) transformants were selected by the method of Hoch (1971) except that chloroform-resistant transformants were selected after 19 h growth on nutrient agar at 42 °C.

Restriction enzymes. *EcoRI* was obtained from M.R.E. Porton, U.K., *HindIII* from Bethesda Research Laboratories (U.K.), and *Sau3A* from New England Bio-Labs. *PstI* was kindly provided by Dr R. A. Flavell, and other enzymes by Dr M. G. Sargent. Enzyme digestion conditions were those of Davis *et al.* (1980).

Gel electrophoresis. Electrophoresis of DNA was generally carried out with a horizontal 0.8% agarose gel in 89 mM-Tris, 89 mM-borate, 2.5 mM-Na₂EDTA, pH 8.2, for 16 h at approximately 1 V cm⁻¹ (Davis *et al.*, 1980). Samples of approximately 1 µg DNA in 25 µl were used. Gels were stained for 30 min with 1 µg ethidium bromide ml⁻¹, and DNA bands identified by UV-fluorescence. Bacteriophage λ DNA samples digested with *EcoRI* and *EcoRI* plus *BamHI* were used as standards (Daniels *et al.*, 1980). To characterize DNA fragments of less than 1 kb, 1.5% and 2.5% agarose gels were used. In this case pBR322 and pHV33 digested with *Sau3A* (Sutcliffe, 1978; Primrose & Ehrlich, 1981) and λ DNA digested with *HindIII* were used as standards. For experiments requiring extraction of DNA bands from gels, 0.8% low gelling temperature agarose (Sigma) was used (Parker & Seed, 1980). Agarose slices 2 mm in width were melted by heating to 65 °C, and then cooled to 37 °C before use.

DNA. (a) DNA was determined using diphenylamine as described by Giles & Myers (1965). (b) Plasmids were

prepared from *B. subtilis* by the method of Gryczan *et al.* (1978). They were prepared from *E. coli* by the method of Guerry *et al.* (1973) after amplification with 300 µg spectinomycin ml⁻¹ (Klein *et al.*, 1980). Plasmid pHM2 was prepared from *E. coli* SL3030 unless stated otherwise. (c) Total DNA (i.e. plasmid plus chromosomal DNA) was prepared by the following method. An exponentially growing culture (5 ml, approx. 0.5 mg dry wt ml⁻¹) was harvested by centrifugation, the pellet resuspended in 0.5 ml 0.15 M-NaCl, 0.1 M-EDTA (pH 8.0) and digested with lysozyme (200 µg ml⁻¹) for 1 h at 37 °C. The preparation was then thoroughly mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol., previously equilibrated with 2 M-Tris base). The mixture was centrifuged for 5 min in an Eppendorf Microfuge, and the aqueous layer removed. This was re-extracted with phenol/chloroform/isoamyl alcohol, after which traces of phenol were removed from the aqueous phase by four extractions with ether. DNA was precipitated by adding alcohol to 70% (v/v), cooling to -70 °C, and then leaving for at least 1 h at -20 °C. DNA was redissolved in 0.015 M-NaCl/0.0015 M-trisodium citrate pH 7.0. (d) λ DNA was prepared following heat induction of strain RB342 (Miller, 1972).

RESULTS

Isolation of strains harbouring plasmid pHM2

The clone bank constructed by Rapoport *et al.* (1979) consisted of *B. subtilis* DNA inserted into pHV33 and maintained in *E. coli*. It was received as a set of 23 pools (A-W), each of 100 clones, and one pool (X) of 48 clones. Plasmid DNA prepared from each pool was used to transform *B. subtilis* SL1013, selecting for Spo⁺ and for Cm^R transformants. Four pools gave Spo⁺ transformants regardless of whether primary selection was for Spo⁺ or Cm^R (Table 2). Pool K was the most productive and was used for further study. The high number of Spo⁺ phenotypes among Cm^R transformants indicated a higher proportion of 'spo-69⁺' clones than would be expected if the plasmid preparation from each pool contained equal proportions from 100 separate clones.

Many more colonies were obtained (in the same cross) when selection was for Spo⁺ rather than Cm^R. Presumably this resulted from more efficient transformation as linear DNA rather than as covalently closed circular plasmid; this was supported by the observation that more than 90% of the transformants selected as Spo⁺ were Cm^S (see below).

Strain SL1060 (*recE4 spo-69*) was transformed with pool K, and a Cm^R transformant was picked, resuspended in SMM (Spizizen's minimal medium; Piggot, 1973), heat treated to kill vegetative bacteria, and inoculated into PAB-Cm. Plasmid DNA was prepared from this culture and used to transform SL1060 to Cm^R. All transformants obtained were Spo⁺. A single colony was re-isolated, and the strain designated SL3024. Plasmid DNA was prepared from SL3024 and used to transform *E. coli* SL2009 to Amp^R. From 500 transformants obtained, a single transformant was re-isolated, checked for Amp^R and Cm^R, and designated SL3030. Plasmid DNA was prepared from SL3030 and shown to have identical mobility to plasmid DNA prepared from SL3024 on electrophoresis in 0.8% agarose gels. This plasmid was designated pHM2. Four other isolates from the original cross with pool K were taken through the same procedure and gave plasmids that were indistinguishable from pHM2 in transforming activity and electrophoretic mobility; these were not studied further.

Table 2. Transformation of SL1013 (*spo-69 lys-3 trpC2*) with plasmid DNA from the clone bank

See text for details of the clone bank. The numbers are colonies obtained from 0.2 ml transformed cultures. Figures in parentheses indicate the number of Spo⁺ colonies among the Cm^R colonies.

DNA	No. of transformants		
	Lys ⁺	Cm ^R	Spo ⁺
Pool J	2	7040 (4060)	106000
Pool K	3	8720 (7570)	267000
Pool N	4	1740 (1260)	13000
Pool P	5	570 (260)	236000
None	2	0	2
168T ⁺	12300	0	7100

Table 3. *Transformation of spo-69 strains with plasmid and with chromosomal DNA*

The numbers are colonies obtained from 0.2 ml transformed cultures. Figures in parentheses indicate the number of Spo⁺ colonies among the Cm^R colonies.

Recipient	Selection	No. of transformants using donor:			
		pHM2	pHV33	168T ⁺	None
SL1013	Cm ^R	2390(2330)	9880	0	0
	Spo ⁺	19560	3	14270	5
	Lys ⁺	0	0	12300	1
SL1060	Cm ^R	255(254)	155	0	0
	Spo ⁺	145	0	4	0

Complementation of spo-69 by pHM2

Plasmid pHM2 prepared from *E. coli* was very efficient at transforming *B. subtilis spo-69* strains to Spo⁺ (Table 3). (The parental plasmid pHV33 did not complement *spo-69*.) Plasmid pHM2 was able to complement *spo-69* in the *recE4* derivative SL1060, towards which chromosomal DNA was inactive as a donor. Presumably, a diffusible 'spo-69' gene product was produced from pHM2, as the *recE4* mutation prevents recombination with the chromosome of the recipient (Mazza & Galizzi, 1978). This was confirmed by studies of SL3024, a Spo⁺ transformant of SL1060. Strain SL3024 was mitomycin C-sensitive, so that the *recE4* mutation was still expressed. The strain carried pHM2, and indeed, was the parental strain for subsequent studies of pHM2. Total DNA prepared from SL3024 (a mixture of chromosomal and plasmid DNA) was used to transform the Spo⁺ Rec⁺ Lys⁻ *B. subtilis* strain BR 151 to Lys⁺. Of 1224 Lys⁺ transformants obtained, 384 were asporogenous. This is consistent with the known linkage of *spo-69* to *lys-3*, and indicates the presence of a cryptic chromosomal *spo-69* mutation in the Spo⁺ strain SL3024. Plasmid pHM2 prepared from *E. coli* did not transform BR151 to Lys⁺ (nor to Spo⁻).

With pHM2 as the donor, almost all transformants selected as Cm^R were also Spo⁺, regardless of whether the recipient was SL1013 or SL1060 (Table 3). Similarly, for SL1060, most transformants selected as Spo⁺ gave Cm^R colonies upon subculture (125 out of 150 tested; a separate cross to that in Table 3). However, 95 out of 100 Spo⁺ transformants of SL1013 proved to be Cm^S on subculture. Presumably integration of part of the plasmid into the chromosome was possible in the latter case, so that plasmid dimers (Canosi *et al.*, 1978) would not be required for transformation; this could account for the great excess of Spo⁺ over Cm^R transformants of SL1013.

The Spo⁺ Cm^R transformants of SL1060 and SL1013 were unstable, and gave Spo⁻ Cm^S bacteria on subculture unless selection for Cm^R was maintained (125 out of 125 tested for SL1060; 55 out of 55 tested for SL1013). This instability was also evident in the initial Spo⁺ selection with SL1060, where many of the colonies were visibly sectorized for the pigment associated with spore formation.

Effect of pHM2 on adjacent mutations

A number of mutations that mapped close together were assigned to the *spoIIA* locus (Piggot & Coote, 1976). A genetic map has recently been described for some of the mutations (Yudkin & Turley, 1981). Mutations *spo-42*, *spo-50* and *sas-1*, which are distal to *lys* on the map, were complemented by pHM2 in a *rec⁺* and a *recE4* genetic background (Table 4). However, mutations *spo-1* and *spo-37*, which are proximal to *lys*, were not complemented. Thus pHM2 carries only part of the *spoIIA* locus (Fig. 1).

Several mutations described by Ionesco & Schaeffer (1968) that were also assigned to the *spoIIA* locus (Piggot & Coote, 1976) were not included in the study of Yudkin & Turley (1981). None of the mutations was complemented by pHM2 (Table 4), although all were efficiently transformed to Spo⁺ by chromosomal DNA (data not shown).

Table 4. Transformation of *spo* mutants with pHM2, selecting for *Cm^R* transformants

The numbers are colonies obtained from 0.2 ml transformed cultures. No *Cm^R* colonies were obtained in the absence of donor DNA.

Recipient		No. of transformants using donor pHM2	
Strain	Relevant genotype	Spo ⁻	Spo ⁺
SL976	<i>spo-42 rec⁺</i>	90	4910
SL986	<i>spo-42 recE4</i>	0	1134
SL450	<i>spo-50 rec⁺</i>	660	5940
SL1067	<i>spo-50 recE4</i>	0	15
SL977	<i>sas-1 rec⁺</i>	140	2190
SL988	<i>sas-1 recE4</i>	0	40
SL437	<i>spo-37 rec⁺</i>	1820	0
SL978	<i>spo-1 rec⁺</i>	544	0
SL979	<i>spo-5 rec⁺</i>	576	0
SL980	<i>spo-4 rec⁺</i>	202	0
SL981	<i>spo-12 rec⁺</i>	252	0
SL983	<i>spo-26 rec⁺</i>	2250	0
SL984	<i>spo-16 rec⁺</i>	4440	0

Fig. 1. Map of the *spoIIA* locus showing the position of the locus present in pHM2. The map is modified from that of Yudkin & Turley (1981). The portion of the locus present in pHM2 is shown by the hatched area.

Restriction map of pHM2

A restriction map for pHM2 (Fig. 2) was constructed from the restriction patterns obtained with *Eco*RI, *Hind*III and *Sal*I, used singly and in combination (Table 5). The double digest patterns were compatible with the map shown, and not with any other arrangement. This interpretation was confirmed by extracting the three largest fragments from *Hind*III and from *Eco*RI digests that had been separated by electrophoresis in low gelling temperature agarose, and analysing the products of restriction with the second enzyme. The sum of fragment sizes for single *Hind*III and *Eco*RI digests indicate a plasmid size of 10.58 kb. We would not have detected the presence of additional fragments that are smaller than 0.18 kb in the single digest; our interpretation of the *Hind*III + *Eco*RI double digest indicates the presence of such a fragment of 0.05 kb. We detected a single *Pst*I site also present in pHV33, and no *Kpn*I, *Xho*I, *Xba*I or *Xma*I sites.

DISCUSSION

Plasmid pHM2 was isolated from the clone bank of Rapoport *et al.* (1979). It consists of a 3.3 kb insert of *B. subtilis* DNA in plasmid pHV33. It confers on *E. coli* resistance to ampicillin and chloramphenicol, and on *B. subtilis* resistance to chloramphenicol. Unless selection is

Fig. 2. Restriction map of pHM2. The *Hind*III site at the top of the map is at the junction of the pC194 and pBR322 portions of the plasmid and is distal to the *Bam*HI site in pHV33 (Ehrlich, 1978). The *Hind*III site is used as the origin for map distances (kb) which are shown inside the innermost circle. Restriction sites for *Hind*III, *Eco*RI, *Sal*I and *Pst*I are shown in the concentric circles. The region of *B. subtilis* DNA is outlined in bold. A, B, C, D and E represent restriction fragments in order of decreasing size.

Table 5. *Sizes (kb) of restriction fragments obtained from pHM2*

Restriction enzyme(s)	Fragment sizes (kb)						
<i>Eco</i> RI	4.9	3.6	1.5	0.38	0.20†		
<i>Hind</i> III	5.9	2.9*	0.85	0.51	0.42†		
<i>Eco</i> RI + <i>Hind</i> III	4.9	2.9*	0.98	0.51	0.42	0.38	0.20†
<i>Eco</i> RI + <i>Sal</i> I	3.7*	3.6	1.5	1.25‡			
<i>Hind</i> III + <i>Sal</i> I	3.7*	2.9*	2.2	0.85‡			

* These fragments were also obtained in comparable digests of pHV33.

† Analysis would not have detected fragments smaller than about 0.18 kb.

‡ Analysis would not have detected fragments smaller than about 0.7 kb.

maintained, pHM2 is rapidly lost from *B. subtilis*. Plasmid pHM2 can complement mutations *spo-42*, *spo-50*, *spo-69* and *sas-1* that have been placed in the *spoIIA* locus. Complementation occurs without recombination with the chromosome, so that a diffusible product effecting the correction must be produced from the plasmid; it is not possible to say whether or not the *spoIIA* promoter is involved.

Mutations in the part of the *spoIIA* locus proximal to *lys* are not complemented by pHM2, even when recombination with the chromosome is possible. Thus the plasmid does not contain the entire *spoIIA* locus. One of the mutations that is not complemented, *spo-1*, is a nonsense mutation (Yudkin & Turley, 1981), so that the non-complemented region must code for a protein. The most likely interpretation of these results is that the *spoIIA* locus contains at least two genes. At least one intact gene, containing *spo-69*, *spo-50*, *spo-42* and *sas-1*, is carried by pHM2 (Fig. 1), while at least one other gene, containing *spo-1* and *spo-37*, is not carried on this plasmid. These genes are presumably adjacent, with a gene junction between *spo-37* and *spo-42*. Such an arrangement is compatible with an operon structure. It is also possible, however, that the locus contains a single structural gene. Plasmid pHM2 would then code for part of the gene, and that part would be able to function in *trans* to complement mutations in the full-length

chromosomal gene. This form of intracistronic complementation, although rare, has been found in the *E. coli* *lac* system (Ullman & Perrin, 1970).

The recombination indices between mutations at opposite ends of the *spoIIA* locus, determined from transformation crosses, varied between 0.14 and 0.21 (Yudkin & Turley, 1981). The authors interpreted the results cautiously, saying, in essence, that one cannot definitely deduce the existence of more than one gene. It can be inferred from these comments that they also considered the results to be compatible with the existence of two genes. Rouyard *et al.* (1967), Coote (1972), and Piggot (1973) have all argued that a recombination index of 0.1 might represent, very approximately, one gene. Taken at face value, this would suggest at least two genes in the *spoIIA* locus. However, gene size is very variable and recombination indices show experimental variation, so that it is not possible to extrapolate with any precision from one gene to another, hence it is not possible to decide the number of genes in the *spoIIA* locus from the recombination indices.

Intercistronic complementation can be distinguished from intracistronic complementation by careful study of the gene product; the former gives a wild-type product whereas the latter does not, its product often being less active and less stable than the wild-type. The immediate gene product (or products) of the *spoIIA* locus is unknown, and has not been assayed quantitatively, but only qualitatively by its ability to function sufficiently for the organism to form a spore. Thus it is not possible to distinguish between intercistronic and intracistronic complementation in this way. We consider it much more likely that the locus contains at least two genes, but cannot formally rule out the possibility of intracistronic complementation.

There have been few reports of the expression of cloned *B. subtilis* *spo* genes in *B. subtilis* (Kawamura *et al.*, 1981; Jayaraman *et al.*, 1981). This is the first report of the cloning of a *spo* gene on a shuttle vector where the gene is expressed in *B. subtilis*, but can also be maintained and studied in *E. coli*. It thus opens the possibility of using the great range of methods for genetic manipulation, notably those employing transposons, that are available in *E. coli*. There are now several reports that plasmids maintained in *B. subtilis* are often less stable than those maintained in *E. coli* (Ehrlich *et al.*, 1981; Krefl & Hughes, 1981); use of the shuttle vector should help circumvent this problem.

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