Regulation of Stage II of Sporulation in *Bacillus subtilis*

By SANDRA CLARKE* AND JOEL MANDELSTAM

Microbiology Unit, Department of Biochemistry, University of Oxford, South Parks Road,
Oxford OX1 3QU, UK

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A mutation, *spo-87*, in the *spo0J* locus of *Bacillus subtilis* allows appreciable transcription of *spoIIA*, *spoIID* and *spoIIG* and later operons, even though most of the cells are morphologically blocked at stage 0 and the incidence of heat-resistant spores is about 1 per 10⁴ cells. This mutation therefore appears to disengage the genetic control of sporulation from the morphological changes to which it should be connected. *spoIIA* and *spoIIG* are transcribed independently of one another. However, the products of both operons are needed for the activation of *spoIID* which occurs later. This indicates a convergence of parallel pathways of operon expression. We have also shown that nonsense mutations in *spoIIAC* (which codes for a sigma factor of RNA polymerase) prevent transcription of *spoIID*; by contrast, missense mutations in the same gene allow transcription of *spoIID*.

INTRODUCTION

Sporulation in bacilli is conventionally divided into six stages based on the appearance of the cells in thin section in the electron microscope (Ryter, 1965; Kay & Warren, 1968). The whole developmental process is controlled by a minimum of 50 genetic loci (Losick *et al.*, 1986); the total number may exceed 60 (Hranueli *et al.*, 1974).

Stage 0 and stage I represent the vegetative cell, and mutations in a number of genetic loci will prevent further development. These are designated *spo0A*, *spo0B*, etc., and it has recently become apparent that most of them, possibly all, are expressed during vegetative growth (Losick *et al.*, 1986; Yamashita *et al.*, 1986) and therefore can be disregarded for our purposes.

Sporulation from the developmental point of view can therefore be considered as beginning at stage II. This begins with the laying down of an asymmetric division septum consisting of a double membrane without appreciable cell wall material. The membrane grows so that the larger of the compartments in the cell engulfs the smaller, which then develops further to form the spore. The series of events between septation and engulfment is regulated by about six known genetic loci (designated *spoIIA*, *spoIIB*, etc.) and it includes some associated biochemical 'markers' of which the most easily measured is the formation of extracellular alkaline phosphatase (Waites *et al.*, 1970; Glenn & Mandelstam, 1971). Mutations in any of the *spoII* loci may either block development after septation or they may cause the formation of a variety of morphological malformations such as multiple septa, premature deposition of cell wall material, etc. These aberrant cell forms have been illustrated in a number of papers (see e.g. Ryter *et al.*, 1966; Coote, 1972*a*; Young, 1976).

One of the loci, *spoIIG*, contains a gene coding for a protein of *M*_r 31 000 (Stragier *et al.*, 1984) which is presumably processed by the protein product of another locus, *spoIIE*, to give a sigma factor for RNA polymerase, σ^{29} , specifically associated with sporulation (Trempey *et al.*, 1985*a,b*).

Another of the loci associated with this stage is *spoIIA*. This has been cloned and sequenced (Fort & Piggot, 1984) and shown to consist of three genes, *spoIIAA*, *spoIIAB* and *spoIIAC*. From measurements of the size of the mRNA produced during sporulation, the three genes have been

shown to be expressed as an operon (Savva & Mandelstam, 1986). Earlier work (Errington & Mandelstam, 1983) had shown that mutations in the A and C genes of this operon led to different phenotypes. Some of them, lying in either of the genes, completely prevented sporulation (asporogeny) and also alkaline phosphatase formation, while others blocked spore formation incompletely (oligosporogeny) and allowed formation of phosphatase at about half the normal rate. Quite closely linked mutations within the same genes could produce either phenotype. In all, 17 mutations have been found which all lie in the A and C genes whereas no sporulation phenotype has so far been associated with the B gene (J. Errington, personal communication).

From the nucleotide sequence of the *spoIIA* operon it appeared that the C gene codes for a protein with a high degree of homology to a variety of sigma factors including the σ^{29} already referred to (Errington *et al.*, 1985; Stragier *et al.*, 1984). In view of a report by Trempey *et al.* (1985a) that expression of *spoIIG* depended on *spoIIA26*, and since we knew that both *spoIIA* and *spoIIG* were needed for expression of *spoIID*, the following provisional order of dependence of sporulation operons was proposed (Clarke *et al.*, 1986):

The work described in the present paper was undertaken because we found that some mutations in *spoIIA*, as we have already mentioned, permitted significant expression of alkaline phosphatase. These mutations also allowed partial expression of the *spoIID* operon. Because of these results we decided to examine mutant *spoIIA26* which Trempey *et al.* (1985a, b) had obtained from the Bacillus Stock Center as a strain carrying a mutation in *spoIIA*. On checking the mutant supplied both by the Center and, kindly, by Dr W. G. Haldenwang, we found that it behaved in all respects as a stage 0 mutant. This clearly cast doubt on our earlier conclusion that *spoIIA* preceded *spoIIG* in a linear sequence.

Our present results show that: (a) *spoIIA* and *spoIIG* are expressed independently of one another and that expression of both operons is necessary for the expression of *spoIID*; (b) a mutation in the *spo0J* locus, *spo-87*, disengages the sequential induction of operons from morphological development.

METHODS

Bacterial strains, phages and plasmids. These are listed in Table 1.

Induction of sporulation and measurement of marker events. Cells were grown and induced to sporulate at 37 °C by the method of Sterlini & Mandelstam (1969). Alkaline phosphatase was measured as described by Glenn & Mandelstam (1971) and modified by Errington & Mandelstam (1983). One unit of enzyme hydrolyses 1 nmol *p*-nitrophenyl phosphate in 1 min at 30 °C. β -Galactosidase was assayed as described by Errington & Mandelstam (1986) or as modified by Clarke *et al.* (1986). One unit hydrolyses 1 nmol MUG (4-methylumbelliferyl β -D-galactoside) min⁻¹. Methods for electron microscopy were those of Kay & Warren (1968).

DNA preparation. Plasmid DNA was extracted from *Escherichia coli* by the alkaline lysis method (Maniatis *et al.*, 1982). Phage ϕ 105 DNA was prepared as described by Jenkinson & Mandelstam (1983). *Bacillus subtilis* chromosomal DNA for transformations was prepared according to Ward & Zahler (1973).

DNA-RNA 'Northern' hybridizations. The choice of probe and its construction were as follows. Plasmid pGSIIG11 (a gift from J. Szulmajster) contains a 1133 bp *Pst*I fragment which codes for a 239 amino acid polypeptide and is able to transform *spoIIG* recipients to Spo⁺ (Stragier *et al.*, 1984). We constructed the plasmid pUC7IIG by subcloning this *Pst*I fragment into the *Pst*I site in plasmid pUC7 (Messing *et al.*, 1981). Ligation, transformation and recombinant screening were as described previously (Clarke *et al.*, 1986). The *Pst*I fragment was then removed from plasmid pUC7IIG with *Eco*RI, which cleaved sites in the polylinker of pUC7, and cloned into phage DI:1t (Flock, 1977). Selection of phage ϕ 105IIG from the bank of recombinant phages was essentially as described elsewhere (Jenkinson & Mandelstam, 1983; Errington, 1984). The resulting recombinant phage ϕ 105IIG was able to transduce to Spo⁺ strains containing mutations *spoIIG41*, *spoIIG55* and *spoIIG6*. These results indicated that plasmid pUC7IIG would be an adequate probe for the detection of *spoIIG* mRNA.

RNA was isolated from *spo* mutants (Table 1) 2.5 h after resuspension in sporulation medium. Trempey *et al.* (1985b) had determined that this would be a time of maximum abundance of *spoIIG* message. Cultures (100 ml) were centrifuged for 3 min and the pellets placed on ice. Cells blocked at stage III or later were washed with 1 ml ice-cold lysozyme solution (10 mg ml⁻¹ in 50 mM-Tris/HCl, pH 8), spun for 30 s in a microfuge, quickly resuspended in 0.5 ml lysozyme solution, frozen in liquid nitrogen and stored at -70 °C. Stage 0 and stage II

Table 1. *Bacterial strains, phages and plasmids*

Bacteria	Genotype	Source/reference
<i>B. subtilis</i> *		
SG46†	<i>lys-1 metC3 tal-1 spoIID::lacZ</i> <i>cat</i> (ϕ 105LD2)	Clarke <i>et al.</i> (1986)
1.5	<i>spoIIA1 trpC2</i>	E1, Piggot (1973)
6.2	<i>spoIIG6 metC3</i>	E10, Piggot (1973)
41.3	<i>spoIIG41 trpC2</i>	NG4.14, Piggot (1973)
42.2	<i>spoIIA42 lys-1</i>	NG6.13, Piggot (1973)
43.2	<i>spo0A43 leu-8</i>	NG6.21, Piggot (1973)
55.2	<i>spoIIG55 trpC2</i>	NG12.12, Piggot (1973)
63.2	<i>spoIIA63 metC3</i>	NG16.2, Piggot (1973)
87.2	<i>spo0J87 leu-8</i>	Hranueli <i>et al.</i> (1974)
93.2	<i>spo0J93 trpC2</i>	Hranueli <i>et al.</i> (1974)
69.1	<i>spoIIA69 trpC2</i>	NG18.6, Piggot (1973)
168†	<i>trpC2</i>	Laboratory stock
560	<i>spoIIA560 hisH2</i>	Errington & Mandelstam (1983)
562.5	<i>spoIIA562 trpC2</i>	Errington & Mandelstam (1983)
563.3	<i>spoIIA563 metC3</i>	Errington & Mandelstam (1983)
564.3	<i>spoIIA564 metC3</i>	Errington & Mandelstam (1983)
MY2000.578	<i>spoIIA578 hisH2 pyrD1</i>	M. Yudkin‡
609	Δ <i>spoIIA trpC2 ilv leu-8</i>	J. Errington‡
IS31	<i>metC3 trpC2 ade spo</i>	BGSC§ strain 26U of Ionesco <i>et al.</i> (1970)
<i>E. coli</i>		
JM103	Δ (<i>lac-pro</i>) <i>thi strA supE endA sbcB15</i> F' <i>traD36 proAB lacF^r ZΔM15</i>	Messing <i>et al.</i> (1981)
Phages	Relevant properties	Reference
ϕ 105LD2	<i>spoIID</i> *, 2 kbp <i>MboI</i> insert in ϕ 105J9	Lopez-Diaz <i>et al.</i> (1986)
ϕ 105DI:1t		Flock (1977)
ϕ 105DS1		Savva & Mandelstam (1984)
ϕ 105IIG	1.1 kbp <i>PstI</i> fragment in ϕ 105DI:1t	This paper
Plasmids	Relevant properties	Reference
pUC7	Ap ^r	Messing <i>et al.</i> (1981)
pSGIIG11	<i>spoIIG</i> clone	Stragier <i>et al.</i> (1984)
pUC7IIG	1.1 kbp <i>PstI</i> fragment in pUC7	This paper

* The other *spo* mutants used are listed in Errington & Mandelstam (1986).

† Strains 168 and SG46 are Spo⁺. The latter carries an intact copy of *spoIID* on the ϕ 105LD2 prophage.

‡ Microbiology Unit, Department of Biochemistry, University of Oxford.

§ Bacillus Genetic Stock Center, The Ohio State University.

mutants were washed with cold Tris/HCl only, since cells blocked early in sporulation lyse easily. Stored cells were thawed at room temperature and added to 3 ml guanidinium solution (6.0 M). Cells at stage III or later were refrozen in liquid nitrogen and rethawed, before the addition of guanidinium, to facilitate breakage of the cells. The RNA was extracted by the guanidinium/caesium chloride method described by Maniatis *et al.* (1982) and electrophoresed through gels containing formaldehyde, also as described by Maniatis *et al.* (1982). The denatured RNA was transferred from the gels to nitrocellulose, the filters prepared and the hybridizations and autoradiography were carried out as described in 'Membrane Transfer and Detection Methods' (Amersham). The DNA probe was nick-translated and labelled with [³²P]dCTP using a kit from Amersham.

Transductions and infection with phage ϕ 105. This was done as described previously (Clarke *et al.*, 1986).

*Transfer of *spo::lacZ* fusions into *spo* mutants.* Two methods were used.

(a) *spoIID*. Competent cells were transformed with DNA from strain SG46, which has a *spoIID::lacZ* transcriptional gene fusion on the chromosome. The resulting transformants would be double mutants since, in addition to the original *spo* mutation, the *spoIID* gene would now be disrupted by the insertion of *lacZ*. The mutant was then complemented by transduction with phage ϕ 105LD2, which carries the complete *spoIID* transcription unit (Clarke *et al.*, 1986), i.e. an intact *spoIID* gene is contained in the prophage and the phenotype is Spo⁺. The transformations, selection of transformants and complementation with phage ϕ 105LD2 were done as described previously (Clarke *et al.*, 1986).

(b) *spoIIA* and *spoIIIC*. Both gene fusions had been cloned into phage $\phi 105$ and were transferred by transduction as described by Errington & Mandelstam (1986) and Turner *et al.* (1986).

RESULTS

Effects of mutations in spo0J on morphological changes and on the expression of operons controlling stage II

The locus *spo0J*, which maps near the replication origin in *B. subtilis* (Trowsdale *et al.*, 1979; Moriya *et al.*, 1985), is defined by two mutations *spo-87* and *spo-93* (Hranueli *et al.*, 1974). The recombination index (RI) between these is 0.39 (Hranueli *et al.*, 1974). On the basis of work done in this laboratory and elsewhere (see e.g. Carlton, 1966; Coote, 1972*b*; Errington & Mandelstam, 1984; Lamont & Mandelstam, 1984) this almost certainly indicates that the mutations lie in separate genes and possibly even in different operons. Certainly, the phenotypes of strains carrying these mutations are distinctly different.

Strain 93.2 is morphologically blocked at stage 0 (Hranueli *et al.*, 1974) and it fails to 'turn on' *spoIIA* (Errington & Mandelstam, 1986), *spoIID* (Fig. 1) or alkaline phosphatase (Fig. 2). Because mutants carrying *spo-93* thus appeared to be like other stage 0 mutants and also because we had difficulty in extracting mRNA from the cells, no serious attempt was made to investigate them more extensively.

Strain 87.2 is also morphologically a stage 0 mutant. Electron microscopic examination shows that, at t_4 , 3% of the cells have reached stage II and 6% have reached a malformed stage III, viz. the engulfed 'prespore' appears to be surrounded by a single instead of a double membrane; the remaining cells are blocked at stage 0. In both mutants, about 1 in 10000 cells makes a heat-resistant spore (J. Errington, personal communication). Although in strain 87.2 the overwhelming majority of the cells remain at stage 0, *lacZ* fusion experiments show that *spoIIIC* and *spoIIA* are both turned on (results not shown) and so is *spoIID* (Fig. 1). Furthermore, 'Northern' hybridization gives a positive signal for *spoIIG* (Table 2). It is also of interest that the formation of alkaline phosphatase, a stage II marker event, begins at about t_1 (Fig. 2), as it does in the wild-type, although the rate of formation is reduced slightly. Nevertheless, both in amount and in the 'take-off point' at about t_1 , the curve for strain 87.2 is clearly distinguishable from the negative result obtained with strain 93.2.

The finding that the expression of sporulation operons is strikingly different in strains carrying two different mutations, apparently in *spo0J*, will be considered later (Discussion).

Effects of mutations in spoIIA on spore formation, sporulation phenotype, the formation of alkaline phosphatase and the expression of spoIID

From the earlier work already referred to (Errington & Mandelstam, 1983) we knew that even closely linked mutations in the A and C genes of the *spoIIA* operon could vary in their effects on spore formation and on the occurrence of biochemical marker events linked to stage II. Since then, the nature of some of these mutations has been determined by sequencing experiments (Yudkin, 1987) and shown to be either missense or nonsense. This made it possible to attempt to make a more rational correlation between the mutations and their phenotypic effects.

We accordingly measured the formation of alkaline phosphatase and the expression of *spoIID* in *spoIIA* mutants carrying the *spoIID::lacZ* transcriptional gene fusion (see Methods).

The more interesting and more interpretable results were those obtained with strains carrying mutations in the C gene, which codes for a sigma factor (Errington *et al.*, 1985). Three of the mutants (strains 1.5, 63.2 and MY2000.578; mutations 1, 63 and 578 in Table 3) carry nonsense mutations (Yudkin, 1987) and in each of them there was no production of alkaline phosphatase (Table 3) and no significant expression of mRNA for *spoIID*. A typical result (Fig. 3) showed that strain 63.2 exhibits the same slow hydrolysis of a β -galactosidase substrate in the control and in the constructed strain carrying a *lacZ* fusion. By contrast, strains (numbers 560, 563.3 and 564.3) carrying missense mutations (Yudkin, 1987) were oligosporogenous (Errington & Mandelstam, 1983) and produced alkaline phosphatase (Table 3). Thus far the results merely confirm those previously reported by Errington & Mandelstam (1983) but, more interestingly,

Fig. 1

Fig. 2

Fig. 1. Production of β -galactosidase when a *spoIID::lacZ* transcriptional fusion was transferred to the chromosomes of sporulation mutants 93.2 and 87.2 by transformation with DNA from strain SG46. This was followed by transduction with phage ϕ 105LD2 to insert an intact copy of the *spoIID* gene. Cells (1 ml) in sporulation medium were assayed for β -galactosidase at hourly intervals from t_0 . ▲, SG46, *spoIID::lacZ* control; △, 87.2 with *spoIID::lacZ* fusion; ○, 93.2 with *spoIID::lacZ* fusion.

Fig. 2. Production of alkaline phosphatase. Cell suspensions (1 ml) in sporulation medium were assayed at hourly intervals from t_0 . ●, 168, wild-type control; △, 87.2; ○, 93.2.

Table 2. *Synthesis of spoIIG mRNA in mutants carrying defects in different spo loci*

The mRNA was detected by 'Northern' hybridization. RNA was isolated 2.5 h after the cells were transferred to sporulation medium and probed for *spoIIG* mRNA as described in Methods. Some of the hybridizations are shown in Fig. 4. +, mRNA present; -, mRNA absent; ND, not determined.

Letter designation of mutated locus	Stage of block:						
	0	II	III	IV	V	VI	Ger
A	-	+	+	+	+		
B	-	+	+	+	+		
C			+	+	+	+	
D	-	+	+	+	+		
E	-	+	+	+	+		+
F	-	+	+	+	+		
G		+		+			
H	-				+		
J { <i>spo</i> -87	+						
<i>spo</i> -93	ND						
K	-

Fig. 3. β -Galactosidase production when a *spoIID::lacZ* transcriptional fusion was transferred to the chromosomes of strains carrying *spoIIA* mutations. This was followed by transduction with phage ϕ 105LD2 to insert an intact copy of the *spoIID* gene. Cells (1 ml) in sporulation medium were assayed at hourly intervals from t_0 . ▲, SG46, *spoIID::lacZ* control; ○, 63.2 control; △, 63.2 with *spoIID::lacZ*; ●, 562.5 with *spoIID::lacZ*; □, 563.3 with *spoIID::lacZ*.

Table 3. *Effects of mutations in the spoIIA operon*

Alkaline phosphatase and the expression of *spoIID* (see also Fig. 3) were measured in *spoIIA* mutants carrying a *spoIID::lacZ* transcriptional gene fusion and an intact copy of the *spoIID* gene in the prophage of ϕ 105LD2.

Mutation (and type*)	Alkaline phosphatase	Expression of <i>spoIID</i>	Sporulation phenotype†
<i>spoIIAA42</i> (missense)	—	—	Osp
<i>spoIIAA69</i> (missense)	—	—	Spo [—]
<i>spoIIAA562</i> (unknown)	+	+	Osp
<i>spoIIAC1</i> (nonsense)	—	—	Spo [—]
<i>spoIIAC63</i> (nonsense)	—	—	Spo [—]
<i>spoIIAC560</i> (missense)	+	+	Osp
<i>spoIIAC563</i> (missense)	+	+	Osp
<i>spoIIAC564</i> (missense)	+	+	Osp
<i>spoIIAC578</i> (missense)	—	—	Spo [—]

* See Yudkin *et al.* (1985); Yudkin (1987).

† See Errington & Mandelstam (1983).

the missense mutations also allow expression of *spoIID*. The result for one of the mutants (563.3, Fig. 3) resembles that obtained with the Spo⁺ control in that there is a sharp rise in the production of mRNA for *spoIID* at about t_1 in contrast to what is found in the nonsense mutant, strain 63.2.

Our observations on the effects of mutations in the A gene of *spoIIA* have been less extensive, and results have so far been obtained with only three mutants (Table 3). These show that interpretation may prove to be more difficult. Thus, strain 562.5 is oligosporogenous and it is positive for the expression of both alkaline phosphatase (Table 3) and *spoIID* (Fig. 3, Table 3). However, strains 42.2 and 69.1, which both carry missense mutations (Yudkin *et al.*, 1985), differ in phenotype. Neither strain produces alkaline phosphatase or mRNA for *spoIID* but one of them is oligosporogenous while the other is asporogenous. Even with this limited analysis it is

clear that the phenotypes of strains carrying missense and nonsense mutations in the A gene will cut across the fairly clear distinction between phenotypes resulting from corresponding changes in the C gene.

Effect of mutation spoIIA26 on stage II events

The strain containing this mutation, originally designated 26U by Ionesco *et al.* (1970), and linked by transduction with phage PBS1 to *lys-1*, was classified as a *spoIIA* mutant and deposited as such in the Bacillus Stock Center (strain IS31). It was then used by Trempey *et al.* (1985*a, b*), who found that it prevented the expression of *spoIIG*. It thus appeared that *spoIIA* was necessary for the expression of *spoIIG* and the provisional order of expression of stage II operons deduced by us and given in the Introduction was based on this finding.

It was obviously of interest to know more of the phenotypic characteristics of strain IS31 and to compare them with those of the *spoIIA* mutants described in Table 3. However, examination of thin sections of the mutant in the electron microscope showed that it is, in fact, blocked at stage 0. In addition, phage ϕ 105DS1, which contains the entire *spoIIA* locus (Savva & Mandelstam, 1984), was unable to transduce strain IS31 to Spo⁺. Thus, morphologically strain IS31 is clearly a stage 0 mutant and we have not attempted to look at its properties more closely. This result alters the order of spore gene expression proposed by Turner *et al.* (1986) and Clarke *et al.* (1986) (see Discussion).

Dependence pattern of spoIIG

Once our experiments had shown that the apparent dependence of *spoIIG* on *spoIIA* was an invalid conclusion based upon the mis-classification of strain IS31 it became necessary to establish the dependence relationships of *spoIIG*. This was done as follows by DNA-RNA hybridization ('Northern' blotting).

RNA was isolated from several strains (numbers MY2000.578, 69.1, 42.2, 562.5 and 563.3) carrying *spoIIA* mutations. All these strains gave positive signals in 'Northern' hybridization with plasmid pUC7IIG as a probe.

Since the *spoIIG* gene (Stragier *et al.*, 1984) and the *spoIIAC* gene (Errington *et al.*, 1985) code for sigma factors with extensive regions of homology we had to check the possibility that a *spoIIG* probe might hybridize to the mRNA for *spoIIAC*. The experiments included two control strains: one of these, strain 63.2, has a nonsense mutation at the beginning of the *spoIIAC* gene (Yudkin, 1987) while strain 609 has a deletion covering the entire *spoIIA* locus (J. Errington, personal communication). RNA from both strains gave positive signals when hybridized to the *spoIIG* probe (Fig. 4). It was thus clear that mRNA for *spoIIG* was indeed synthesized in mutant strains that were unable to generate a functional mRNA corresponding to *spoIIAC*.

Some examples of the results of 'Northern' hybridization experiments are shown in Fig. 4. Strains carrying mutations *spo0A43* and *spoIIG55* were used as negative and positive controls. Although there was some smearing of the signals, presumably as a result of degradation of mRNA, the distinction between positive and negative strains was easily made.

The expression of *spoIIG* in a variety of other sporulation mutants was then investigated (see Table 2). Expression of the message is dependent upon all the stage 0 loci with the exception, already mentioned, of one of the *spo0J* mutations (*spo-87*). It is apparently not dependent on any other sporulation operon and it is even expressed in strains carrying deletions of *spoIIA* (see previous paragraph) as well as in other strains carrying missense mutations in *spoIIA*.

DISCUSSION

Dissociation of morphological changes from the expression of sporulation operons by a mutation in spo0J

Most of the stage 0 operons are not only expressed during vegetative growth, but mutations in them affect a number of apparently unconnected physiological properties of the cells such as competence in genetic transformation (Spizizen, 1965) and susceptibility to infection by bacteriophages (Ito & Spizizen, 1972), etc., and cause a total block which affects sporulation

Fig. 4. 'Northern' hybridization analysis showing the presence of *spoIIG* mRNA in *spoIIA* mutants. Each lane contains about 8 µg RNA, extracted 2.5 h after cells were transferred to sporulation medium, and probed with plasmid pUC7IIG. RNA from strains 55.2 and 43.2 was used as positive and negative control, respectively. Lanes: 1, 63.2 *spoIIAC63*; 2, 562.5 *spoIIAA562*; 3, 609 Δ *spoIIA*; 4, 43.2 *spo0A43*; 5, 55.2 *spoIIG55*; 6, 563.3 *spoIIAC563*; 7, DNA size markers. Plasmid pUC7IIG was cut with *Pst*I. The bottom band is the 1.13 kbp insert which contains the *spoIIG* gene.

both morphologically and in the expression of sporulation-specific operons (see for example Errington & Mandelstam, 1986; Clarke *et al.*, 1986; Turner *et al.*, 1986; Savva & Mandelstam, 1986). It was therefore proposed (Clarke *et al.*, 1986) that, in general, the stage 0 operons could be excluded from a consideration of the developmental stages leading to spore formation. Instead, they could be regarded as affecting the general make-up of the cell so that it is changed in a number of diverse properties which include, among other things, the ability to form an asymmetric septum and the ability to initiate the dependent sequence of operon expression that controls the development of the spore. The exception appears to be *spo0J*, which is defined by two mutations, *spo-87* and *spo-93*. These, by genetic mapping, are so far apart that they are almost certainly in separate genes (see above).

Mutation *spo-87* is of interest because in the majority of cells carrying it (>90%) sporulation is blocked morphologically at stage 0. It was therefore curious that Trempey *et al.* (1985a) and Rong *et al.* (1986) should respectively have found that *spoIIG* and *spoIID* were transcribed in strains carrying *spo-87*.

The findings presented here show that this mutation also allows the expression of another operon specifically concerned with stage II, viz. *spoIIA*. It also allows the formation of alkaline phosphatase, which is characteristically associated with stage II (Fig. 2), the expression of *spoIIIC*, and also the synthesis of dipicolinic acid (J. Errington, personal communication), which in normal development is a stage V event. It is thus apparent that in strain 87.2 the expression of the dependent sequence of sporulation operons has been set in train but that it is dissociated from the morphological changes that should accompany it.

Independent expression of two operons, spoIIA and spoIIG, coding for sigma factors

The analysis of mutants carrying partial or total deletions of *spoIIAC* (see Results) shows that they can nevertheless express *spoIIG*. Conversely, mutations in *spoIIG* do not prevent expression

Fig. 5. Tentative scheme illustrating the order of expression of operons concerned in stage II of sporulation. In general, mutations in any of the stage 0 operons prevent morphological changes and the transcription of stage II operons. The exception is a mutation in the *spo0J* locus which blocks septum formation, but allows transcription of sporulation operons to be set in train (see text). *spoIIA* codes for a sigma factor; *spoIIG* codes for a 'pre-sigma' factor which is presumed to be converted to the active protein, σ^{29} , by the intervention of the protein product of *spoIIE*. The genes coding for sigma factors thus seem to be turned on independently and the products of both are needed for the transcription of *spoIID* and of 'later' operons (see text). Brackets indicate that the order of operon expression has not yet been resolved and question marks indicate the possible intervention of undiscovered stage II operons.

of *spoIIA* (Savva & Mandelstam, 1986; Errington & Mandelstam, 1986). We are therefore left with the curious finding that two genes coding for very closely homologous sigma factors (both with M_r values of about 29000) are expressed independently at the very outset of induction of sporulation.

The two genes, or their protein products, have further properties in common. (a) Their expression is dependent on an intact set of stage 0 genes, but (b) the expression of both – and that of 'later' operons in the dependent sequence – occurs in a strain carrying mutation *spo-87*. (c) The protein products of both genes are needed for the expression of *spoIID* (Clarke *et al.*, 1986).

In molecular terms it is difficult to conceive that the transcription of *spoIID* depends on the simultaneous presence of two RNA polymerases containing slightly different sigma factors. Instead, it is more likely that one or both of these loci promotes the expression of stage II operons which are as yet unrecognized and that the products of these play a part in the transcription of *spoIID*. The latter has the characteristic properties of a DNA-binding protein and presumably it plays a regulatory role (Holland *et al.*, 1987). This would in turn lead to the transcription of operons that regulate the later stages of sporulation (Turner *et al.*, 1986).

In summary, we offer a tentative scheme for the expression of the operons regulating stage II of sporulation (Fig. 5).

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