Effects of Transition Mutations in the Regulatory Locus spoIIA on the Incidence of Sporulation in Bacillus subtilis

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We have determined the changes in DNA sequence corresponding to three mutations in the promoter-proximal open reading frame of spoIIA, a locus that regulates sporulation in Bacillus subtilis. All three mutations prevent the synthesis of two sporulation-associated enzymes, but they differ in their effects on spore incidence. We now find that mutation spo-42, which allows spores to be produced at a low incidence, is a transition that changes Gly95 to Asp in the protein encoded by the open reading frame. Mutation spo-69, which blocks sporulation entirely, consists of two transitions: these change Gly62 to Asp and Ala116 to Thr. Mutation sas-1, which partially suppresses spo-69, is also a transition: this changes residue 62 (which had become Asp as a result of the spo-69 mutation) to Asn.

INTRODUCTION

When Bacillus subtilis cells are transferred from a growth medium to a medium with a poor carbon source, they begin a process of differentiation that leads, after about 8 h at 37°C, to the formation of heat-resistant spores (for a review, see Young & Mandelstam, 1979). During this time many specific genes, which were not transcribed during vegetative growth, become expressed in a regulated sequence (Linn & Losick, 1976; Ollington & Losick, 1981; Yudkin et al., 1982). One of the means by which the regulation of gene expression is thought to be achieved involves changes in the specificity of RNA polymerase as development of the spore proceeds (Losick, 1982). However, our understanding of the details of the control systems in sporulation is very fragmentary.

Several dozen genetic loci are necessary for sporulation, as can be inferred from the effect of mutations that block the sequence (reviewed by Piggot & Coote, 1976). We have been studying one of these loci, spoIIA, which we have reason to believe is important in controlling gene expression in sporulation. Different closely-linked mutations in spoIIA have different phenotypic effects in cells placed under conditions that induce sporulation in the wild-type: some abolish and others permit the synthesis of two enzymes, alkaline phosphatase and DNAase, that are associated with stage II of sporulation (Errington & Mandelstam, 1983); some prevent sporulation altogether and others allow a few spores to be made (Piggot, 1973); and one mutation causes the progress of sporulation to be slowed (Yudkin & Turley, 1980).

The nucleotide sequence of the spoIIA region has recently been determined in this laboratory (Fort & Piggot, 1984). It contains three open reading frames (ORFs), the promoter-proximal of which (ORF 1) is expected to encode a protein of 117 amino-acid residues with an isoelectric point of about 5-8.

We have now begun a study of the DNA sequences of different spoIIA mutants, and in this paper we report the identification of the nucleotide changes corresponding to three mutations in

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Abbreviation: ORF, open reading frame.
ORF 1. Each of these mutations prevents the synthesis of the sporulation-associated alkaline phosphatase and DNAase, but the three differ in the extent to which they affect sporulation. Mutation spo-42 allows spores to be produced at about $10^{-4}$ of the wild-type incidence (Yudkin & Turley, 1981), spo-69 entirely abolishes sporulation (Piggot, 1973) and sas-1 partially reverts spo-69 (Yudkin & Turley, 1980).

METHODS

Plasmids. Plasmid pHM2 (Liu et al., 1982) carries a part of the spoIIA region including the whole of ORF 1. When this plasmid is inserted into a strain that bears a mutation in the corresponding region of spoIIA, the mutation is readily transferred to the plasmid (Chak et al., 1982). Dr P. J. Piggot (NIMR, Mill Hill, London, UK) kindly gave us plasmids prepared in this way, that carried either spo-69 or spo-42 or sas-1. We transformed each plasmid into Escherichia coli strain DH1 (Hanahan, 1983), with selection for ampicillin resistance. Plasmids were prepared from strain DH1 by the method of Guerry et al. (1973) after amplification with spectinomycin (Klein et al., 1980), and purified on a gradient of CsCl.

We proved that each plasmid preparation was free of spo+ DNA by showing that it was unable to transform a B. subtilis strain carrying the corresponding spo mutation to Spo+. We confirmed the identity of the spo mutation in each preparation by transforming a spo+ strain to chloramphenicol resistance, recovering the spo mutation from the plasmid on to the chromosome, allowing loss of the plasmid, and characterizing the resulting spo strain by appropriate physiological and genetic tests including back-crosses.

Each plasmid was restricted, and the 0.67 kbp EcoRI-BglII fragment that carries ORF 1 of spoIIA (Fort & Piggot, 1984) was purified on low-melting-point agarose and cloned into M13mp9 (Messing & Vieira, 1982) that had been cut with EcoRI and BamHI. In some experiments the EcoRI-BglII fragments were further digested with AhoI and cloned into the SmaI site of M13mp9.

Chemicals and enzymes. The materials needed for sequencing by the method of Sanger et al. (1977) were obtained from Amersham, and the procedures were as described by the supplier. Restriction enzymes were obtained from BRL, and used according to the directions of the supplier. Low-melting-point agarose was also from BRL.

RESULTS

Sequencing procedure. All the mutations that we wished to study are in ORF 1 of spoIIA, which is entirely contained within a 0.67 kbp fragment bounded by EcoRI and BglII sites (see Fig. 1). By cloning this fragment from plasmids to which the mutations had been transferred, sequencing from the EcoRI site, and comparing the results with the wild-type sequence, we were able to identify the mutations. They all lay between two AluI sites, of which one is near the middle of ORF 1 and the other is early in ORF 2 (Fig. 1). We could thus confirm the identification of the mutations by subcloning the AluI fragment in each case in the two possible orientations and then sequencing both DNA strands. We have thus identified each mutation by three separate cloning and sequencing procedures.

Mutation spo-42. This mutation is a transition at position 670 from the nucleotide pair G·C to A·T (Fig. 2). This transition changes the codon GGT (Gly) to GAT (Asp), at residue 95 in the ORF 1 protein.

Mutation spo-69. To our surprise, spo-69 turned out to be a double mutation, containing two G·C to A·T transitions. The first, at position 571, changes the codon for residue 62 from GGC (Gly) to GAC (Asp). The second, at position 732, changes the codon for residue 116 from GCA (Ala) to ACA (Thr).

Mutation sas-1. This mutation, which is also a transition from G·C to A·T, is at position 570, within the same codon as the first of the spo-69 changes. Thus in the sas-1 mutant the codon for residue 62 reads AAC (Asn), as compared with GGC in the wild-type and GAC in the spo-69 mutant.

DISCUSSION

Although we have discovered two mutations, separated by 160 base pairs, in spo-69, we believe that it is the first of these, at position 571, that is responsible for the Spo- phenotype. Our reasons are these. First, we would expect a change from glycine to aspartic acid, which would introduce an additional negative charge, to have a more dramatic effect on the ORF 1 protein.
than a change from alanine to threonine. Secondly, we have tried, by using DNA from a spo-69 lys+ strain to transform a spo+ lys strain and selecting for Lys+, to obtain recombinants that carry only the mutation at position 732; but even though we can calculate that several of these should have arisen we have been unable to find any recombinants with a distinctive Spo phenotype (M. D. Yudkin, unpublished work). Thirdly, genetic mapping unequivocally places spo-69 on the lys-distal side of spo-42 (Yudkin & Turley, 1981), which suggests that what is being mapped in these experiments is the mutation at position 571. We therefore conclude that it is this mutation that is responsible for the Spo phenotype; presumably the additional change at position 732 occurred in the original N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis that produced spo-69, or, possibly, by subsequent genetic drift in the inactivated gene.

The fact that mutations spo-42 and spo-69 both lead to the replacement of glycine residues by aspartic acid residues is presumably due to chance, but it allows us to focus attention on the way in which the position of the amino-acid change affects the phenotype. Strains carrying spo-42 produce spores at about 10⁻⁴ of the wild-type incidence; in other words the mutation is leaky (Yudkin & Turley, 1981). Strains carrying spo-69, by contrast, produce no detectable spores whatever (Yudkin & Turley, 1981), so we may conclude that residue 62 is more critical to the function of the ORF 1 protein than is residue 95.

The importance of amino-acid residue 62 is further emphasized by our finding that mutation sas-2, which was selected as leading to partial reversion of spo-69, results in a change in this residue from aspartic acid to asparagine. Strains carrying sas-1 make spores at about 10⁻⁴ of the wild-type incidence (Yudkin & Turley, 1980). Thus, although the protein has its full function with glycine at position 62, a change to asparagine allows partial function; on the other hand, aspartic acid at the same position totally abolishes activity, no doubt because of its charge.

Since sas-1 was selected from a mutant carrying spo-69, which has a mutation at nucleotide 732, one might ask why the sas-1 sequence is wild-type at this position. In order to answer this question, we have to trace the history of sas-1. From a strain carrying spo-69, Yudkin & Turley (1980) selected a partial revertant which proved still to harbour spo-69. This revertant was presumed by Yudkin & Turley (1980) to be a double mutant, spo-69 sas-1; in the light of the present results, which show that spo-69 is itself a double mutant, we now believe that the
revertant actually contained three mutations, at positions 570, 571 and 732. When DNA from this revertant was used to transform a spo+ strain, recombinants were obtained at a frequency of about 0.2%, which were presumed to carry sas-I alone. It is this genotype that we have now sequenced: since it contains transitions at positions 570 and 571 but not at position 732 we conclude that the last of these mutations was crossed out in the transformation. Thus the designation sas-I of Yudkin & Turley (1980) actually refers to transitions in two adjacent base pairs.

Six different mutations in ORF 1 have been isolated on the basis of their phenotypic effects on sporulation and have been genetically mapped in this laboratory (Yudkin & Turley, 1981; Errington & Mandelstam, 1983). Of these six, spo-69 is the closest to that region of ORF 1 that determines the N-terminus of the protein. The fact that we have now found that spo-69 alters position 62 of a 117-residue protein suggests that mutations in the N-terminal half of the protein may be without effect on sporulation and may therefore have escaped detection.

These studies have allowed us to make a start in relating structure to function in a protein that is involved in the regulation of spore development. We are now investigating other mutants, including some that prevent sporulation but allow the synthesis of the sporulation-associated alkaline phosphatase and DNAase to continue.

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REFERENCES


