CATABOLITE RESISTANT MUTANTS OF *BACILLUS SUBTILIS*

By

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ABSTRACT

Mutations that confer resistance to catabolites in sporulation (crs) are located in six distinct loci on the chromosome of Bacillus subtilis. One of the crs mutations, crsE1, is found in the rpoBC operon which codes for β and β' subunits of RNA polymerase. Some stv and std mutations in the same operon also confer partial resistance to glucose. Another mutation, crsA47, is located in the gene for the σ-factor of RNA polymerase. These findings indicate that certain mutations in RNA polymerase can alter the response of cells to the inhibitory effect of catabolites on sporulation.

The crs mutants differ from each other in the growth characteristic and glucose utilization. Specific activities of IMP dehydrogenase and alkaline phosphatase vary widely among the crs mutants. These results suggest that the growth characteristic and the enzymes examined are not closely related to catabolite resistance in sporulation.

The crs mutants are resistant to at least one of the membrane-affecting agents, cerulenin, ethanol and NaCl in sporulation. This suggests that the membrane and its associated functions are important in initiation of sporulation.

It is also suggested that functions affected in the crs mutants are related to the membrane.

Mutations scal9, rfml1, eryl and relA can offset the catabolite resistance in some of the crs mutants. Possibly, these suppressors suppress the catabolite resistance by affecting the membrane or by causing a metabolic imbalance which affects membrane functions.

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Chapter I

INTRODUCTION

Bacterial sporulation

Under certain conditions, species of Bacillus, Clostridium, Sporolactobacillus, Desulfotomaculum, Sporosarcina and Oscillospira are able to form endospores (Buchanan and Gibbons 1974). The spores are in a dormant state and usually they are resistant to the lethal effect of heat, drying, organic solvents and other toxic agents. The existence of bacterial spores was first noticed as early as the 1870's when Pasteur's studies on spontaneous generation were challenged by the observation that infusions of hay after boiling failed to remain sterile. Tyndall and Cohn then demonstrated that the organisms which survived boiling were endospores of the hay bacillus (Bacillus subtilis) (quoted in Davis et al. 1973). In 1877, Cohn and Koch described the formation of spores in Bacillus anthracis, the pathogen of anthrax (quoted in Davis et al. 1973). Since then, studies on the properties of spores and the process of sporulation have been carried out in many laboratories. Most investigations have been undertaken with species of Bacillus and Clostridium because of their importance in medicine and in

various industries. More recently, sporulation has been considered as a simple model system of cellular differentiation, and consequently the morphological, biochemical and genetic aspects of bacterial sporulation have been intensively investigated in the past three decades (see reviews by Piggot and Coote 1976; Sonenshein and Campbell 1978; Young and Mandelstam 1979; Freese 1981; Losick 1982).

1. Morphological changes during sporulation

The development of the bacterial spore is associated with a sequence of morphological changes which have been investigated by the electron microscopy. Although these changes are continuous, it is convenient to divide the process of sporulation into several stages as described by Young and Fitz-James (1959a, 1959b) and Ryter (1965).

Vegetative cells are referred to as in stage 0 in which chromosomes are seen as discrete, compact and roughly spherical bodies. Sporulation usually starts in a large fraction of cells at the time when exponential growth ends or when cells growing exponentially in a nutrient-rich medium are transferred into a nutrient-poor medium. This time is taken as the time of induction of sporulation (t_0) and the hourly periods thereafter are designated as t_1, t_2 and so on (Schaeffer et al. 1963; Sterlini and Mandelstam 1969).

Stage I (t_0 - t_1) is indicated by the formation of an axial nuclear filament from the fusion of two or more nuclear bodies observed in the vegetative cells. The chromosomes subsequently separate and one of them migrates to a position near the pole of the cell. Since no mutants unable to form the axial nuclear filament have been found and because similar filaments are often observed in cells of non-spore-forming bacteria, it is suggested that the axial filament may represent a non-specific response of the DNA under certain conditions (quoted in Robinson and Spotts 1983).

Stage II (t_1 - t_2) begins when the cytoplasmic membrane invaginates asymmetrically at one end of the cell to form a prespore which contains a copy of the chromosome. The development of the prespore septum differs from the cell-division septum in that it is sub-polar rather than central and that only a small amount of cell wall materials are deposited between its two membrane layers. The prespore septum is formed by de novo membrane synthesis rather than by the rearrangement of pre-existing membrane.

During stage III (t_2 - t_3), a free forespore is formed within the mother cell by bulging of the double-layered prespore septum into the cytoplasm of the mother cell, followed by movement of the points of attachment of the ends of the septum toward the pole of the cell. The forespore protoplast thus formed is surrounded by two membrane layers

which are oriented with opposite polarity. After the formation of the forespore, the cell is committed to continue subsequent development. After this time, sporulation will proceed normally even if the sporulating cells are transferred into a fresh medium. It has been suggested that the phenomenon of commitment may be related to the absence of active transport of nutrients through the forespore double-layered membrane (Freese et al. 1970).

Stage IV (t_3 - t_4) is marked by the synthesis of a germ cell wall and a cortex layer between the two membrane layers around the forespore protoplast. At the end of stage IV, the forespore becomes visible under the phase-contrast microscope as a grey body at one end of the cell.

The subsequent formation of a spore coat around the cortex is referred to as stage V (t_4 - $t_{5.5}$). During this stage, the spores become phase-bright as seen under the phase-contrast microscope. After the development of refractility, the spores acquire resistance to a number of organic solvents such as chloroform and octanol. The resistance to octanol has been associated with the development of the spore coat (Ryter 1965).

Stage VI ($t_{5.5}$ - t_7) involves the maturation of the spores, at which time the spores undergo a number of changes to develop their characteristic resistance to heat and other

physical conditions. Finally, the mother cell is lysed and the spore is released. This stage is referred to as stage VII (after t_7).

2. Biochemical changes during sporulation

The morphological changes during sporulation are associated with a number of sequentially occuring biochemical events (Warren 1968; Schaeffer 1969; Waites et al. 1970). One of the earliest biochemical events is the production of both extracellular and intracellular proteases shortly after t_0 (Millet 1970; Prestidge et al. 1971; Reysset and Millet 1972; Cheng and Aronson 1977). The extracellular protease activity includes a serine protease with an alkaline optimum pH and a metalloprotease with a neutral optimum pH (Millet 1970; Prestidge et al. 1971). The metalloprotease has been thought to be unrelated to sporulation since mutants lacking this activity sporulate normally (Michel and Millet 1970). On the other hand, the serine protease seems to be intimately related to initiation of sporulation. Many B. subtilis mutants whose sporulation is blocked at stage 0 (spo0 mutants) are usually unable to produce the serine protease (see review by Schaeffer 1969). Inhibitors of serine protease, phenylmethylsulphonyl fluoride and m-amino benzeneboronic acid can suppress sporulation but not vegetative growth of

wild-type cells (Dancer and Mandelstam 1975; Geele et al. 1975). However, it has been reported that certain mutants of B. subtilis deficient in extracellular protease are able to sporulate (quoted in Szulmajster 1982). Several other Bacillus species do not produce detectable amount of this enzyme and yet they form spores (Levisohn and Aronson 1967; Millet and Aubert 1969; Slapikoff et al. 1971). Consequently, it is suggested that the extracellular serine protease may not be essential for sporulation (Piggot and Coote 1976; Young and Mandelstam 1979; Szulmajster 1982). The intracellular serine protease is thought to be involved in protein turnover that occurs during early stages of sporulation (Forster and Perry 1954; Kornberg et al. 1968). This activity may also be involved in the post-translational modification of proteins required for sporulation (Sadoff et al. 1970; Cheng and Aronson 1977; Szulmajster 1982). Kerjan et al. (1979) have isolated a mutant which has a mutation in the structural gene of an intracellular protease. This mutant is temperature-sensitive in both sporulation and the production of intracellular protease while the production of extracellular protease is unchanged. Revertants of this mutant which regain the ability to produce intracellular protease at high temperature also regain the ability to sporulate under the same condition. Thus these authors suggest that the intracellular protease plays an important role in sporulation.

The production of antibiotics soon after t_0 is another biochemical event that occurs at the beginning of sporulation (Abraham and Florey 1949). The antibiotics produced by B. subtilis inhibit growth of Staphylococcus aureus (Schaeffer et al. 1963) and B. subtilis strain H (Spizizen 1965). Since many stage 0 mutants are deficient in antibiotic production, the possible role of antibiotics in initiation of sporulation has been proposed (see reviews by Schaeffer 1969; Katz and Demain 1977). However, mutants that are unable to produce the antibiotics but are able to sporulate have been isolated from Bacillus licheniformis (Haavick and Thomassen 1973). From the above observations, it seems that synthesis of the antibiotics is not obligatory for sporulation.

It has been known that changes in the activity of many enzymes occur during early stages of sporulation. The activity of enzymes involved in the tricarboxylic acid cycle (TCA cycle) increases significantly at the beginning of sporulation (Hanson et al. 1964). The elevated TCA cycle enzyme activities maintain the concentration of ATP required for sporulation (Freese 1972; Ohné and Rutberg 1976). Many mutations affecting TCA cycle enzymes block sporulation at stage 0 (Klofat et al. 1969; Szulmajster and Hanson 1965; Freese and Marks 1973). These mutations are not sporulation-specific since they also affect vegetative growth under cer-

tain conditions. In some cases sporulation of these mutants can be restored by the addition of TCA cycle intermediates to the cultures (Ohné and Rutberg 1976) or by the partial inhibition of synthesis of guanine nucleotides (Freese et al. 1979c). Freese and Fortnagel (1969) have reported that the addition of acetate but not other carbon sources can restore sporulation of a number of stage 0 mutants that lack the pyruvate dehydrogenase complex. These authors suggest that an elevated concentration of acetyl-Coenzyme A is more important for sporulation than for growth.

Enzyme activities in the respiratory chain also undergo a significant increase soon after t_0 (Taber et al. 1972; Felix and Lundgren 1973; Weber and Broadbent 1975). This increase is necessary for providing a sufficient amount of ATP required for sporulation. Mutants lacking cytochromes are usually asporogenous (Taber et al. 1972; Taber and Freese 1974). Certain stage 0 mutants do not show the wild-type pattern of an increase in cytochromes and menaquinone after the end of exponential growth (Taber et al. 1972; Weber and Broadbent 1975). Lang et al. (1972) have found that inhibition of the electron transport chain by the addition of cyanide has little effect on growth of Bacillus cereus but strongly inhibits sporulation. These observations suggest that a typical electron transport system is not required for vegetative growth but is absolutely required for sporulation

(Fortnagel and Freese 1968; Lang et al. 1972).

A rapid decrease in the activity of several enzymes occurs during the initial stage of sporulation in Bacillus species (Deutscher and Kornberg 1968; Bernlohr and Gray 1969). The relationship between the decrease in enzyme activities and initiation of sporulation is unclear. Bernlohr and Gray (1969) suggest that the decrease in activities of threonine dehydrogenase, aspartokinase and pyruvate kinase in B. licheniformis is related to the physiological conditions of cells and not to the process of sporulation. When cells enter the stationary phase, the synthesis of aspartate transcarbamylase stops; this is followed by the inactivation of the existing enzyme (Maurizi and Switzer 1978). Since the cessation of aspartate transcarbamylase synthesis is also observed in a number of stage 0 mutants, it is not clear whether the decrease in the enzyme activity is related to sporulation (Maurizi and Switzer 1978). The decrease in inosine monophosphate (IMP) dehydrogenase, the first enzyme for the synthesis of guanine monophosphate (GMP) from IMP, has also been reported by Deutscher and Kornberg (1968). They have found that, in an asporogenous B. subtilis mutant the activity of aspartate transcarbamylase decreases as in the wild-type strains, where as IMP dehydrogenase remains at a relatively high level for up to 10 h of incubation. The same mutant is also unable to show a decrease in adenylate

kinase activity which occurs in wild-type cells (Deutscher and Kornberg 1968). These investigators have not been able to decide whether the regulation of IMP dehydrogenase and adenylate kinase is directly related to sporulation, or the decrease in the levels of these two enzymes observed during sporulation is simply due to proteolytic degradation.

The synthesis of alkaline phosphatase has been regarded as a sporulation-associated event (Young and Mandelstam 1979). The activity of this enzyme begins to increase during stage II of sporulation (Waites et al. 1970). This increase takes place even in the presence of excess inorganic phosphate, the condition which represses the synthesis of alkaline phosphatase during vegetative growth (Anagnostopoulos 1960; Glenn and Mandelstam 1971; Ichikawa and Freese 1974). Mutants that are unable to produce this enzyme during vegetative growth can produce it normally during sporulation (Ichikawa and Freese 1974). Some spo mutants are unable to synthesize this enzyme (see review by Piggot and Coote 1976). Piggot and Taylor (1977) have reported that the ability to synthesize alkaline phosphatase in these spo mutants can be restored by introducing other mutations designated as sap. However, the spo sap double mutants are still asprogenous. Isolation of mutants with the altered structural gene of alkaline phosphatase would be helpful to determine whether this enzyme is necessary for

sporulation. However, this type of mutant has not been reported so far.

During stage II, an extracellular DNase is produced which is active against double-stranded DNA (Akrigg 1978). Stage 0 and stage II mutants which are unable to produce alkaline phosphatase are also unable to produce the extracellular DNase (Akrigg and Mandelstam 1978). Another enzyme, glucose dehydrogenase is synthesized during the transition from stage III to stage IV (Waites et al. 1970; Coote 1972). Mutants blocked at or before stage II do not synthesize this enzyme. It is not clear whether the extracellular DNase and glucose dehydrogenase have a specific role in spore formation, although the latter enzyme is known to play a role in spore germination (Prasad et al. 1972).

The assembly of the spore coat at stage V involves the modification of pre-existing precursors (Cheng and Aronson 1977; Pandey and Aronson 1979). Antisera made against an alkali-soluble coat protein fraction of B. subtilis can precipitate proteins from cells at stage II of sporulation, indicating that the synthesis of the spore coat precursors starts as early as stage II (Wood 1972). This conclusion is supported by the observation that spore coat proteins are synthesized in mutants whose sporulation is blocked at stage II (Wood 1972).

Dipicolinic acid (DPA) is synthesized at stage V

(Vinter 1962). The location of DPA in spores is indefinite (discussed in Pearce and Fitz-James 1971). All mutants blocked before or at stage IV and some stage V mutants cannot produce DPA (see review by Piggot and Coote 1976). It is generally believed that DPA is related to heat-resistance of spores (see review by Hanson et al. 1970). Murrell (1981) suggests that the heat-resistance may be the result of interactions of DPA or Ca-DPA complex with macromolecules such as DNA and proteins. On the other hand, some DPA-less mutants have been shown to be able to form heat-resistant spores (Hanson et al. 1972; Zytkovicz and Halvorson 1972). The spores produced by these mutants tend to lose heat-resistance upon storage and do not germinate in various liquid media. Hanson et al. (1972) suggest that DPA may be required for maintaining the established heat-resistance and for normal germination.

3. Genetic studies on sporulation

Spizizen (1958) has reported the DNA-mediated transformation for tryptophan auxotrophy in B. subtilis. Takahashi (1961) has discovered bacteriophage PBS1 and successfully used this phage in transduction crosses. The size of transforming DNA fragments varies from about 10^6 to 10^7 daltons, which is less than 0.5% of the entire chromosome of B. subtilis. The small size of transforming DNA allows fine ge-

netic analysis of closely linked mutant genes. At the same time, this situation makes the construction of a continuous linkage map extremely difficult. Transducing particles of phage PBS1, on the other hand, transfer as much as 5-8% of the donor chromosome into recipient cells (Yamagishi and Takahashi 1968a). Phage PBS1 has been useful in constructing the circular genetic map of B. subtilis (Lepesant-Kejzlarova et al. 1975). By the use of transformation and transduction, numerous mutations affecting sporulation as well as those affecting vegetative functions have been genetically characterized (see review by Henner and Hoch 1980).

Genetic analyses involving spo mutations were first carried out by transformation. The spo mutations are defined as mutations that affect sporulation but do not affect vegetative growth. It has been shown that there are a number of spo loci that control different stages of sporulation (Schaeffer et al. 1959; Schaeffer and Ionesco 1960; Spizizen 1961). Takahashi (1965a, 1965b) has studied spo mutations by PBS1-mediated transduction and by transformation and established the first linkage map for spore genes in B. subtilis. Since then at least 33 spo loci have been identified on the chromosome of B. subtilis (see review by Piggot and Coote 1976). Among them, nine are spo0 loci that control the earliest stage (stage 0 to I) of sporulation. These loci have been designated as spo0A, spo0B and so on according

to the phenotypes of the mutants and their map positions. Other spo loci, including seven stage II, five stage III, seven stage IV and five stage V loci have been also identified (Piggot and Coote 1976).

As spo0 mutants have a block at the earliest stage of sporulation, studies with these mutants have been extensively carried out to obtain information on initiation of sporulation. Mutations in the spo0 loci often result in pleiotropic phenotypes. As discussed earlier, many spo0 mutants are not only defective in sporulation but are also unable to produce proteases and antibiotics. They also fail to show an increase in the enzymatic activities of the TCA cycle and the respiratory chain. In addition, many spo0 mutants appear to be hypersensitive to antibiotics produced by wild-type strains of the same species (Ito et al. 1971). They are also hypersensitive to polymixin B (Guespin-Michel 1971a; Michel and Millet 1970) which is known to bind preferentially to phospholipids (Sebek 1967). Many spo0 mutants are sensitive to several bacteriophages that are unable to infect wild-type cells (Ito and Spizizen 1971). According to these authors, this trait may be related to the membrane since it has been shown that in Escherichia coli certain membrane proteins are involved in the restriction of phage T4 (Frankel et al. 1968).

The loss of transformability is often observed in

spo0 mutants (Spizizen 1965). In B. subtilis, mesosomes which are membranous vesicles, are considered to be involved in the establishment of the competent stage for transformation (Vermeulen and Venema 1974). Smith et al. (1983) have recently shown that a membrane-bound protein is responsible for the binding of transforming DNA and is present only in competent cells.

Moreover, spo0 mutants are often found to be hyper-producers of a membrane-bound enzyme, nitrate reductase A (Bohin et al. 1976a). During the last two generations of growth, the phospholipid composition of spo0 mutants appears to be different from that of wild-type strains (Rigomier et al. 1974). All these results suggest that the pleiotropic phenotypes of the spo0 mutants may have been resulted from certain alterations in the membrane (Ito et al. 1971; Ito and Spizizen 1971; Schaeffer et al. 1971; Bohin et al. 1976a).

The functions affected by the spo0 mutations have been investigated by isolating their suppressor mutations. Some spo0A mutations can be suppressed by either a nonsense suppressor (Hoch and Spizizen 1969) or a missense suppressor (Hoch 1971). These authors suggest that the spo0A gene product may be a protein which is required for the positive control of sporulation. In agreement with this view, Trowsdale et al. (1978a) have reported that the spo0A mutations are recessive to the wild-type allele in the spo0A/spo$^+$

partial diploids. The spo0B gene product has also been suggested to be a protein from the observations that the spo0B$^+$ allele is dominant over the spo0B mutations in partial diploids (Hoch et al. 1978) and that some mutations in the spo0B locus result in Spo^{ts} phenotype (Hirochika et al. 1981). Recently, the spo0B$^+$ gene has been cloned by several groups of investigators (Hirochika et al. 1981; Bonamy and Szulmajster 1982; Ferrari et al. 1982). The cloned spo0B$^+$ gene codes for a polypeptide which has a molecular weight of 39,000 (Hirochika et al. 1981) or 24,000 (Bonamy and Szulmajster 1982). This protein is synthesized in both vegetative cells and sporulating cells (Hirochika et al. 1981).

Several investigators have studied partial suppressor mutations which overcome some of the pleiotropic effects of spo0A and spo0B mutations. The partial suppressor mutations include cpsX (Guespin-Michel 1971a, 1971b), abs (Ito et al. 1971), tol (Ito 1973) and abr (Trowsdale et al. 1978b). These mutations are capable of reverting the pleiotropic phenotypes of the spo0 mutants but not the asporogenous phenotype. Shiflett and Hoch (1978) have found that the spo0A abrB double mutants differ from the parental spo0A strains in one of several ribosomal proteins. These authors suggest that the spo0A strains may be defective in translation of mRNA that are related to the pleiotropic phenotypes such as the production of proteases and antibiotics.

This defect can be overcome by abrB mutations which alter the ribosomal protein (Shiflett and Hoch 1978).

Dubnau et al. (1981) have cloned a DNA fragment from B. licheniformis which can complement spo0H mutations of B. subtilis. The cloned DNA fragment codes for a 27,000-dalton polypeptide. It is not clear whether the spo0H gene of B. subtilis is homologous to this cloned DNA fragment of B. licheniformis (Dubnau et al. 1981).

In spite of the above investigations, the exact functions affected by the spo0 mutations and the identity of the spo0 gene products are still unknown.

4. Transcriptional and translational control of sporulation

(1). Transcriptional control

It has been found that some bacteriophages such as β3 and φe can grow in vegetatively growing cells but not in sporulating cells (Yehle and Doi 1967; Sonenshein and Roscoe 1969). Losick and Sonenshein (1969) have observed that RNA polymerase from vegetative cells can transcribe φe DNA, while that from sporulating cells fails to do so. These authors suggest that there is a change in template specificity of RNA polymerase occuring at the beginning of sporulation.

Sporulating cells are known to synthesize new species of mRNA that are absent during vegetative growth (Doi and

Igarashi 1964; Aronson 1965; Yamagishi and Takahashi 1968b; Di Cioccio and Strauss 1973; Sumida-Yazumoto and Doi 1974). However, a significant number of genes that are expressed during the vegetative growth phase continue to be transcribed during sporulation (Sumida-Yazumoto and Doi 1974). Doi (1977) suggests that two types of transcription apparatus may exist simultaneously in sporulating cells: one type is capable of transcribing vegetative genes and the other type is capable of transcribing sporulation-specific genes.

The core enzyme of RNA polymerase from sporulating cells is found to be associated with several types of sporulation-specific polypeptides that are not normally found in vegetative cells (Nishimoto and Takahashi 1974; Duie et al. 1974; Linn et al. 1975; Fukuda and Doi 1977). Fukuda and Doi (1977) have observed that a species of RNA polymerase from sporulating cells contains a polypeptide with molecular weight of 28,000 (δ^1) or 20,000 (δ^2). The transcriptional activities of δ-containing enzyme are higher than those of vegetative holoenzymes when assayed with poly (d(AT)) as template, indicating a regulatory role of the δ-factors (Fukuda and Doi 1977). These authors suggest that the new forms of RNA polymerase recognize a new set of genes that are necessary for the process of sporulation.

Losick and his co-workers (Haldenwang and Losick 1979; Haldenwang et al. 1981) have identified three forms of

B. subtilis sigma factors (σ^{55}, σ^{37} and σ^{29}) which confer distinct promoter specificity to the RNA polymerase. The RNA polymerase containing σ^{37} or σ^{29} are able to transcribe two cloned sporulation-related genes (0.4kb and spoVC) in vitro, whereas the enzyme containing vegetative sigma factor (σ^{55}) fails to do so (Haldenwang and Losick 1979; Haldenwang et al. 1981; Moran et al. 1981a). The nucleotide sequences of the promoter regions in the cloned 0.4kb and spoVC genes differ significantly from those of the cloned vegetative genes of B. subtilis (Moran et al. 1981a, 1981b). They are also different from the E. coli promoters (Rosenberg and Court 1979).

Evidence for the transcriptional control of sporulation has been also obtained from the studies on RNA polymerase mutants of B. subtilis that are resistant to antibiotics such as rifamycin, streptovaricin and streptolydigin (Haworth and Brown 1973; Sonenshein et al. 1974). The rifamycin resistance (rfm) and streptovaricin resistance (stv) mutations are located in the rpoB gene which codes for the β subunit of RNA polymerase (Halling et al. 1977). On the other hand, the rpoC gene which codes for the β' subunit is affected by the streptolydigin resistance (std) mutations (Halling et al. 1978). Doi (1982) suggests that the rpoB and the rpoC genes form an operon which regulates RNA polymerase synthesis in B. subtilis. While most rfm, stv

and std mutants sporulate normally, some mutants are found to be asporogenous (Spo^-) or unable to sporulate at restrictive temperatures (Spo^{ts}) (Doi 1977; Wayne et al. 1981). Hirochika and Kabayashi (1978) have reported that a group of rfm mutations can suppress the Spo^{ts} phenotype of a fusidic acid resistant mutant which has an altered elongation factor G for protein synthesis.

(2). Translational control

The existence of translational control of gene expression in E. coli and other bacteria (Steitz 1969; Hsu and Weiss 1969; Dube and Rudland 1970) has prompted a search for a similar mechanism which might occur during sporulation. Many mutants of B. subtilis that are resistant to ribosome-affecting antibiotics such as streptomycin (Leighton 1974), erythromycin (Domoto et al. 1975), spectinomycin (Graham and Bott 1975) and fusidic acid (Fortnagel and Bergmann 1973) are deficient in sporulation. The deficiencies have been associated with changes in ribosomal proteins (Leighton 1974; Domoto et al. 1975; Graham and Bott 1975) or in elongation factor G (Fortnagel and Bergmann 1973). Fortnagel et al. (1975) have reported that ribosomes from vegetative and sporulating cells differ in structure and sensitivity to fusidic acid. Chambliss and Legault-Demare (1975) have found that one of the initiation factors for protein synthesis

from sporulating cells has a lower activity than that from vegetative cells in translating mRNA synthesized after SPO-1 infection. These observations suggest that changes in translational specificity might occur during sporulation. However, there is no direct evidence showing that ribosomes from sporulating cells can preferentially translate sporulation-specific mRNA. Aronson and Del Valle (1964) have reported the presence of long-life mRNA in sporulating cells. However, other investigators have not been able to confirm the existence of such long-life mRNA (Szulmajster et al. 1963; Leighton and Doi 1971). As for tRNA, no difference has been found between sporulating cells and vegetative cells in isoaccepting species of tRNA (Vold 1975).

Recently, RNA molecules that carry a polyadenylated tail of 160-180 nucleotides at their 3'-end (poly(A) RNA), have been found in sporulating cells of Bacillus polymyxa (Kaur and Jayaraman 1979) and B. subtilis (Graef-Dodds and Chambliss 1978; Kerjan and Szulmajster 1980). Szulmajster (1982) has observed that the proportion of poly(A) RNA increases significantly during sporulation in B. subtilis. This increase is sporulation-specific since a stage 0 mutant fails to do so (Szulmajster 1982). The populations of poly(A) RNA are heterogenous in size and have messenger activities (Szulmajster 1982). Kerjan et al. (1982) have found that a cloned DNA fragment hybridizeable to poly(A) RNA is able to

transform a spo0B mutant to spo0B$^+$. From these results, Szulmajster (1982) suggests that poly(A) RNA may be transcribed from DNA sequences that contain spo genes. Jayaraman and Murthy (1982) have proposed that in B. polymyxa early spore genes may be enriched in d(AT) sequences and located in the membrane-bound DNA. Szulmajster (1982) speculates that poly(A) may be necessary for protecting the newly synthesized mRNA during sporulation.

5. Initiation of sporulation

(1). Modification of RNA polymerase

As discussed earlier, several lines of evidence suggest the existence of transcriptional control of gene expression during sporulation. Modified forms of RNA polymerase which contain various sigma-like factors are suggested to play an important role in the sequential expression of sporulation-specific genes (Fukuda and Doi 1977; Haldenwang and Losick 1979; Haldenwang et al. 1981).

Losick (1981) suggests that spo0 gene products may be involved in the modification of RNA polymerase. A single mutation in the spo0 genes often causes pleiotropic effects and changes the synthesis of several proteins (Brehm et al. 1975). Losick (1981) proposes that the spo0 gene products may interact with the transcriptional or translational ma-

chineries of vegetative cells. According to the same author, the spo0 gene products, which are "vegetative proteins" are components of a pathway that senses the nutritional condition of cells. In vegetative cells, one of the sigma factors, σ^{55} or σ^{37} binds to the core enzyme of RNA polymerase. Upon nutrient deprivation, the spo0 gene products turn on early sporulation genes by direct or indirect interaction with the σ^{37}-RNA polymerase or with the promoters that can be recognized by the σ^{37}-RNA polymerase. The spo0 gene products would then remove σ^{55} and σ^{37} from the core enzyme and replace them with a new sigma factor, σ^{29} which allows the transcription of genes that are expressed in stages II and III. As Losick (1981) has pointed out, a test of this model requires information on the genes that code for different sigma factors and the mode of action of the spo0 gene products.

(2). Highly phosphorylated nucleotides

Rhaese et al. (1975) have identified a number of highly phosphorylated adenine nucleotides that appear during the onset of sporulation of B. subtilis. Two of these nucleotides, ppApp and pppApp, are synthesized by ribosomes of sporulating cells but not by those of spo0 mutant cells (Rhaese et al. 1977). Another nucleotide, pppAppp, is synthesized by a membrane-bound synthetase (Rhaese and

Groscurth 1976). The pppAppp synthetase is present during vegetative growth but does not start to synthesize pppAppp until phosphorylated metabolites of glucose are used up (Rhaese and Groscurth 1976). Mutants which accumulate sugar-phosphates and fail to sporulate cannot synthesize pppAppp. These results have led Rhaese and Groscurth (1976) to propose that the synthesis of pppAppp upon nutrient deficiencies triggers initiation of sporulation. Since mutants carrying spo0F mutations are unable to produce this nucleotide, Rhaese et al. (1977) suggest that the spo0F gene may code for the synthetase for pppAppp. However, the purified preparation of pppAppp synthetase has not been available for its characterization. Several other laboratories have not been able to detect pppAppp under their sporulation conditions (Nishino et al. 1979; Smith et al. 1980). Recently, Kawamura and Saito (1983) have shown that an intergenic suppressor mutation, sof1, can fully restore sporulation of a mutant in which the spo0F gene promoter and a part of the structural gene have been removed by a 0.7kb-deletion.

(3). Deprivation of guanine nucleotides and stringent response

Freese and co-workers (Mitani et al. 1977; Freese et al. 1978) have found that leaky purine-requiring mutants sporulate well in the presence of excess glucose, ammonia and

phosphate when exogenous purine supply is limited. These authors have also found that the inhibitory effect of glucose, ammonia and phosphate can be overcome by the addition of hadacidin and decoyinine which are inhibitors of purine nucleotide synthesis. With the exception of 6-azauracil, none of the inhibitors of pyrimidine nucleotide synthesis has the stimulatory effect on sporulation (Freese et al. 1978). Although 6-azauracil inhibits de novo UMP synthesis, it also causes a decrease in GTP level (Lopez et al. 1979). Hadacidin, an inhibitor of AMP synthesis, lowers the concentrations of both ATP and GTP in wild-type cells (Lopez et al. 1979). However, decoyinine, an inhibitor of GMP synthesis, decreases the concentration of GTP, while increasing the concentration of ATP (Lopez et al. 1979). Under various conditions known to initiate sporulation, there is always a decrease in the level of GDP and GTP. The level of adenosine, cytidine and uridine di- and triphosphates increases in some cases and decreases in other cases under the same conditions (Lopez et al. 1979, 1981a). Freese and co-workers conclude that a decrease in the level of GDP and GTP is important in initiation of sporulation

Lopez et al. (1981b) have reported that sporulation can be initiated by the stringent response under conditions of partial deprivation of amino acids and the initiation of sporulation is accompanied by a decrease in GTP level. Re-

laxed mutants (relA) which are unable to produce the stringent response do not sporulate under the same conditions, neither show the decrease in the level of GTP (Lopez et al. 1981b). These authors suggest that the initiation of sporulation by the stringent response is due to the decrease of GTP level rather than the accumulation of ppGpp and pppGpp. Ochi and Freese (1983) have shown that a number of ribosome-affecting antibiotics such as chloramphenicol and fusidic acid inhibit sporulation by preventing the stringent response. Again, the inhibitory effect of these antibiotics can be counteracted by the addition of decoyinine (Ochi and Freese 1983).

(4). Importance of the membrane

It has been known that the activity of membrane-bound electron transport system increases significantly at the onset of sporulation (Felix and Lundgren 1973; Taber et al. 1972; Weber and Broadbent 1975). Mutants which do not show such an increase are often asporogenous (Taber et al. 1972; Taber and Freese 1974). In agreement with these results, changes in the composition of membrane proteins during early stages of sporulation have been also detected (Goldman 1976; Andreoli et al. 1981).

The pleiotropic phenotypes of spo0 mutants discussed earlier have been suggested to be the result of alterations in the membrane (Schaeffer et al. 1971; Rigomier et al.

1974). The abs mutations, which can suppress some of the pleiotropic phenotypes of spo0A mutants have been found to affect the eletrophoretic pattern of membrane proteins (Ito and Spizizen 1972). Bohin et al. (1976b) have shown that ethanol, which changes the phospholipids of the membrane (Rigomier et al. 1980), blocks sporulation at stage 0. Bohin and Lubochinsky (1982) suggest that successful initiation of sporulation depends on the proper rearrangement and interaction of membrane constituents. Furthermore, Wayne et al. (1981) have shown that the Spo^{ts} phenotype induced by antibiotic cerulenin, an inhibitor of fatty acid synthesis (Wille et al. 1975), can be physiologically suppressed by the addition of ribose or synthetic lipids into the culture media. The Spo^{ts} phenotype of various mutants which are affected in RNA polymerase or ribosomes can also be suppressed by various carbon sources or synthetic lipids (Wayne and Leighton 1981). The same authors suggest that a correct state of membrane synthesis or functions is a critical requirement for initiation of sporulation.

6. Inhibition of sporulation by catabolites

(1). Effect of catabolites on developmental processes

Relatively high concentrations of glucose and other carbon sources are known to suppress sporulation of B.

subtilis (Schaeffer et al. 1965; Freese et al. 1970). Schaeffer et al. (1965) have found that cells sporulate during the exponential growth phase at a probability which depends on the nature of both the carbon and the nitrogen sources present in the medium. These authors suggest that sporulation is repressed by carbon- and nitrogen-containing catabolites which control the synthesis of a sporulation-specific protease. Freese and co-workers (Freese et al. 1972, 1974; Oh et al. 1973) have reported that mutants which accumulate various catabolites in cells because of the subsequent metabolic block by mutations fail to sporulate. At least three compounds, glucose-6-phosphate, glycerolphosphate, and a heitherto unidentified compound derived from malate have been found to suppress sporulation.

Glucose is also known to inhibit other bacterial developmental processes such as flagellar formation (Adler and Templeton 1967). The formation of flagella requires 25 specific gene products (for review see Silverman and Simon 1977). Mutant strains of E. coli and Salmonella typhimurium defective in adenylate cyclase are unable to form flagella (Yokota and Gots 1970). Dobrogosz and Hamilton (1971) have observed that the addition of cAMP to wild-type cultures of E. coli grown in the presence of glucose can release the catabolite repression of flagellar formation. Thus it appears that cAMP plays an important role in flagellar formation.

Silverman and Simon (1974) have reported that a cfs mutation allows the formation of flagella under the condition of catabolite repression. These authors suggest that the cfs mutation permits the transcription of its adjacent flaI gene even when the cAMP-CAP complex is absent. According to these authors, the flaI gene product may in turn induce the expression of other flagella-specific genes.

The relation between catabolite repression of inducible enzymes and inhibition of sporulation by catabolites in B. subtilis has been studied by several investigators. Coote (1974) has found that, when chromosome replication of a thymine-requiring mutant is blocked by thymine starvation, the induction of several enzymes such as histidase, sucrase and α-glucosidase can proceed normally while sporulation cannot be initiated. The author concludes that the mechanism for overcoming the inhibition of sporulation differs from that involved in overcoming the repression of inducible enzymes. Takahashi (1979) has shown that some crs mutants that are resistant to glucose and other carbon sources for sporulation are not resistant in induction of acetoin dehydrogenase. It is concluded that there is no necessary close relation between catabolite repression of this enzyme and that of sporulation (Takahashi 1979). Lopez et al. (1980) have reported that, in the presence of excess glucose, the addition of decoyinine can initiate sporulation but cannot over-

come the catabolite repression of inducible enzymes such as acetoin dehydrogenase, inositol dehydrogenase and sorbitol dehydrogenase. These authors conclude that the mechanism which represses the syntheses of these enzymes differs from that which inhibits sporulation.

(2). Effect of catabolites on induction of enzymes

Makman and Sutherland (1965) have found that the intracellular concentration of cAMP in E. coli is low when the synthesis of inducible enzymes is repressed by glucose. This observation suggests a positive control by cAMP in the regulation of inducible enzyme synthesis. The action of cAMP requires a protein factor, CAP (Emmer et al. 1970). It is generally believed that the cAMP-CAP complex binds to the promoter site of catabolite sensitive operons, which allows the transcription of these operons. The enzyme for the synthesis of cAMP, adenylate cyclase, is associated with the membrane. Glucose and other carbon sources may lower the intracellular concentration of cAMP by inhibiting the activity of this enzyme.

In B. subtilis, the synthesis of an inducible enzyme, acetoin dehydrogenase is repressed by various phosphorylated intermediates (Lopez and Thoms 1977). Recently, Fisher and Sonenshein (1984) have isolated a mutant which lacks glutamine synthetase. In this mutant the syntheses of

several enzymes such as aconitase, histidase and citrate synthase are relatively insensitive to catabolite repression. Fisher and Sonenshein (1984) suggest that either the glutamine synthetase protein or the product, glutamine is involved in the regulation of several metabolic pathways in B. subtilis. Fisher and Magasanik (1984) have also isolated a mutant which can synthesize aconitase and histidase in the presence of glucose. Since the levels of pyruvate, 2-ketoglutarate and oxaloacetate in this mutant are lower than those in wild-type strains, these authors suggest that these metabolites are involved in mediating catabolite repression of enzyme synthesis in B. subtilis (Fisher and Magasanik 1984).

The foregoing observations indicate that glucose and other carbon sources have a profound effect on both developmental processes and enzyme synthesis in bacteria. However, in spite of repeated attempts made by several investigators, the presence of cAMP and adenylate cyclase has not been demonstrated in Bacillus species (Ide 1971; Setlow 1973; Bernlohr et al. 1974). At present, the mechanism by which the catabolites inhibit developmental processes and enzyme synthesis in B. subtilis is still not clearly understood.

(3). Catabolite resistant mutants for sporulation

In B. subtilis, mutants that are able to sporulate in the presence of glucose and other carbon sources have been

isolated in several laboratories. Freese et al. (1970) have described a mutant which is capable of sporulating in the presence of glucose. This mutant lacks phosphoenolpyruvate transferase activity and cannot grow with fructose as the sole carbon source. Ohné and Rutberg (1976) have isolated a mutant whose sporulation is resistant to glucose as well as malate. These authors suggest that glucose and malate inhibit sporulation possibly through a common mechanism. Ito and Spizizen (1973) have isolated a number of catA mutants that are insensitive to glucose in sporulation. Dod and Balassa (1978) suggest that their scoC mutants may be identical with the catA mutants. The $sacU^h$ mutant reported by Kunst et al. (1974) is capable of sporulating in the presence of glucose. The catA, scoC and $sacU^h$ mutants described by the above authors are found to be hyperproducers of protease.

To obtain information on initiation of sporulation, Takahashi (1979) has isolated a number of B. subtilis mutants (crs mutants) which are able to sporulate in the presence of glucose and other carbon sources at 55mM. Takahashi (1979) has found that mutants which are isolated with fructose, gluconate, mannose, ribose and malate as selective agent are also resistant to glucose. Similarly, mutants that are isolated with glucose as selective agent are also resistant to carbon sources other than glucose. Takahashi and MacKenzie (1982) have shown that the catabolites block sporulation of

wild-type strains at stage 0, while the crs mutants show the normal morphological changes of spore formation. These authors therefore suggest that functions which are inhibited in wild-type cells by the catabolites but not in the crs mutants may be important for initiation of sporulation.

To elucidate the mechanism by which crs mutants show resistance to catabolites, the effect of a number of inhibitory agents on sporulation of these mutants has been investigated (Takahashi and MacKenzie 1982). It has been found that, while sporulation of wild-type strains is inhibited by novobiocin and acridine orange, some of the crs mutants are resistant to these agents. Since single mutations carried by these mutants confer resistance to catabolites as well as to these two agents, Takahashi and MacKenzie (1982) suggest that there may be some common paths between the effect of catabolites and that of these agents.

Takahashi (1979) has found that, although most of the crs mutants are resistant to glucose for sporulation, only those which are isolated with fructose or glucose as selective agent are able to synthesize acetoin dehydrogenase in the presence of glucose. The crs mutants differ from each other also in their sensitivity to the inhibitory effect of novobiocin and acridine orange on sporulation (Takahashi and MacKenzie 1982).

The present thesis includes the following investi-

gations on the crs mutants:

(1) To establish relations between the crs mutants, genetic mapping of the mutants was carried out by transduction and transformation.

(2) Biochemical and physiological characterization of the crs mutants were undertaken to identify the functions that are affected in the mutant strains.

(3) Since suppressor mutations are useful in determining the function affected by a mutation and interrelations between groups of mutations, attempts were made to isolate mutants which showed the Crs^S (sensitive to catabolites for sporulation) phenotype due to the presence of a suppressor mutation. The effect of the suppressor mutations and other mutations such as rfml1, rev4, eryl, and relA on sporulation of the crs mutants was also investigated.

Chapter II

MATERIALS AND METHODS

1. Symbols and abbreviations

The units of length, weight, volume and time were abbreviated as in the Canadian Journal of Microbiology.

The following abbreviations were used to designate mutants that are resistant to various carbon sources for sporulation (Takahashi 1979):

GLU	glucose
GLN	gluconate
FRU	fructose
MAL	malate
MAN	mannose
RIB	ribose

Symbols to designate the genotypes and the phenotypes of mutant strains were according to Demerec *et al.* (1965). The following symbols were used to designate the genotypes of mutant strains used in this study:

crsA, *B*, *C*, *D*, *E*, *F*	catabolite-resistance for sporulation
sca	suppressor for *crs* mutations
rfm	rifamycin resistance

std	streptolydigin-resistance
stv	streptovaricin-resistance

The following symbols were used to abbreviate various phenotypes:

Crs^{R} (S)	catabolite resistant (sensitive) for sporulation
Ery^{R} (S)	erythromycin resistant (sensitive)
Rfm^{R} (S)	rifamycin resistant (sensitive)
Stv^{R} (S)	streptovaricin resistant (sensitive)
Std^{R} (S)	streptolydigin resistant (sensitive)
Str^{R} (S)	streptomycin resistant (sensitive)
Spo^{+}	sporogenous
Spo^{-}	asporogenous
Spo^{ts}	temperature sensitive for sporulation

2. Bacteriophage and bacterial strains

Bacteriophage PBS1 (Takahashi 1961) was obtained from our laboratory stock. The phage was maintained in spores of *B. subtilis* according to Takahashi (1964).

Strains of *B. subtilis* used in this study are listed in Table 1. Sporogenous strains were maintained on

Table 1. Bacterial strains used

Strain	Genotye	Source
SB19E		I. Takahashi
WS	strA1	I. Takahashi
WSnal	strA1 nalA1	I. Takahashi
Kit-1	trpC2 purA16 cysA14	R. A. Dedonder
Kit-2	trpC2 aroI906 dal1 purB33	R. A. Dedonder
Kit-3	trpC2 trel2 metC3 glyB133	R. A. Dedonder
Kit-4	trpC2 pyrD1 ilvA1 thyA1 thyB1	R. A. Dedonder
Kit-5	trpC2 gltA292	R. A. Dedonder
Kit-6	trpC2 aroD120 lys1	R. A. Dedonder
Kit-7	trpC2 leuA8 aroG932 a1d1	R. A. Dedonder
Kit-8	trpC2 hisA1 thr5	R. A. Dedonder
Kit-9	trpC2 sacA321 ctrA47	R. A. Dedonder
GLU-47	strA1 crsA47	I. Takahashi
GLU-1	crsA1	I. Takahashi
FRU-4	crsA4	I. Takahashi
GLU-40	crsB40	I. Takahashi
RIB-2	crsC1	I. Takahashi
GLN-2	crsC2	I. Takahashi
MAN-1	crsD1 crsE1	I. Takahashi

Table 1. Continued

Strain	Genotype	Source
MAN-A1	crsD1	MAN-1 td CS20
MAN-B1	crsE1	MAN-1 td CS21
MAL-4	crsF4	I. Takahashi
1A177	ery-1 guaA1	BGSC
1A187	trpC2 ksgA618 thyA1 thyB1	BGSC
1A248	trpC2 rfm500	BGSC
1S17	trpC2 spo0E11 pheA1	BGSC
CB400	ery1	T. Leighton
CB2401	ery1 rev4	T. Leighton
IS56	relA lys1 trpC2	I. Smith
CS1	trpC2 crsA47	GLU-47 td Kit-6
CS2	trpC2 aroD120 crsA47	GLU-47 td Kit-6
CS3	trpC2 lys1 crsA47	GLU-47 td Kit-6
CS4	trpC2 crsA1	GLU-1 td Kit-6
CS5	trpC2 crsA4	FRU-4 td Kit-6
CS13	trpC2 metC3 trel2	Kit-3 Gly^+ revertant
CS14	trpC2 pyrD1 trel2	Kit-4 Thy^+ Ilv^+ revertant
CS19	purA16 cysA14	Kit-1 Trp^+ revertant
CS20	purA16	WS tf CS19
CS21	cysA14	WS tf CS19

Table 1. Continued

Strain	Genotype	Source
CS22		WS $\xrightarrow{tf}$ CS21
CS23	strA1	WS $\xrightarrow{tf}$ CS21
CS24	crsD1 crsE1	MAN-1 $\xrightarrow{td}$ CS21
CS27	cysA14 crsD1	MAN-1 $\xrightarrow{td}$ CS19
CS28	purA16 crsD1	MAL-A1 $\xrightarrow{td}$ CS19
CS29	strA1 crsE1	WS $\xrightarrow{tf}$ MAN-B1
CS30	rfm1 crsE1	Spontaneous Rfm^R mutant of MAN-B1
CS31	rfm11 crsE1	Spontaneous Rfm^R mutant of MAN-B1
CS33	rfm11	CS31 $\xrightarrow{tf}$ CS22
CS35	rfm11 strA1	CS31 $\xrightarrow{tf}$ CS23
CS36	rfm11 strA1 crsA4	CS35 $\xrightarrow{td}$ FRU-4
CS37	rfm11 strA1 crsC1	CS35 $\xrightarrow{td}$ RIB-2
CS38	rfm11 strA1 crsF4	CS35 $\xrightarrow{td}$ MAL-4
CS39	rfm11 strA1 crsD1	CS35 $\xrightarrow{td}$ MAN-A1
CS40	rfm11 crsA47	CS33 $\xrightarrow{td}$ GLU-47
CS41	rfm11 strA1 crsC2	CS35 $\xrightarrow{td}$ GLN-2
CS70	strA1 crsA47 trpC2 aroD120 lys1	GLU-47 $\xrightarrow{tf}$ Kit-6
CS71	metC3 crsF4 trel2	MAL-4 $\xrightarrow{tf}$ CS13
CS75	std4 crsE1	Spontaneous Std^R mutant of MAN-B1

Table 1. Continued

Strain	Genotype	Source
CS88	stv23	Spontaneous Stv^R mutant of CS22
CS90	stv27	Spontaneous Stv^R mutant of CS22
CS122	strA1 crsA47 sca19	MMS mutagenesis of GLU-47
CS123	strA1 crsA47 sca27	MMS mutagenesis of GLU-47
CS145	strA1 crsA47 a1d1 aroG932 trpC2	GLU-47 tf Kit-7
CS146	strA1 crsA47 thr5 hisA1 trpC2	GLU-47 tf Kit-8
CS225	stv53 strA1	EMS mutagenesis of WS
CS249	crsA1 sca19	CS122 tf CS4
CS250	crsA4 sca19	CS122 tf CS5
CS256	std201 strA1	EMS mutagenesis of WS
CS277	stv53 std201 strA1	CS225 tf CS256
CS294	stv339 syd201 strA1	EMS mutagenesis of CS256
CS298	strA1 crsC1 thr5	CS146 tf RIB-2
CS299	strA1 crsE1 thr5	CS146 tf MAN-B1
CS300	strA1 crsF4 thr5	CS146 tf MAL-4
CS302	strA1 crsD1	CS146 tf MAN-A1
CS304	strA1 sca19 crsF4	CS122 td CS300
CS305	strA1 crsF4	CS122 td CS300

Table 1. Continued

Strain	Genotype	Source
CS306	strA1 sca19 crsC1	CS122 td CS298
CS309	strA1 sca19 crsE1	CS122 td CS299
CS318	strA1 sca27 crsA47 aroD120 trpC2	CS123 tf CS70
CS319	strA1 sca19 crsA47 aroD120 trpC2	CS122 tf CS70
CS320	strA1 sca19 trpC2	WS td CS319
CS327	strA1 rev4	WS td CB2401
CS330	eryl crsA47	CB400 td GLU-47
CS331	eryl crsC1	CB400 td RIB-2
CS332	eryl crsD1	CB400 td CS302
CS337	eryl crsF4	CB400 td CS305
CS338	eryl std4 crsE1	CB400 tf CS75
CS340	strA1 sca19 cysA14	CS21 tf CS320
CS341	sca19 crsD1	MAN-A1 td CS340
CS365	relA crsD1 trpC2	MAN-1 tf IS56
CS370	crsE1 leuA8 trpC2	MAN-1 tf Kit-7
CS372	leuA8 trpC2	MAN-1 tf Kit-7
CS373	crsC1 leuA8 trpC2	RIB-2 tf Kit-7
CS374	strA1 leuA8 trpC2	WS tf Kit-7
CS375	strA1 crsF4 leuA8	CS374 tf MAL-4
CS377	strA1 crsA47 leuA8 trpC2	CS374 tf CS1

Table 1. Continued

Strain	Genotype	Source
CS384	*relA trpC2*	IS56 $\xrightarrow{td}$ CS372
CS387	*relA crsC1 trpC2*	IS56 $\xrightarrow{td}$ CS373
CS391	*relA crsE1 trpC2*	IS56 $\xrightarrow{td}$ CS370
CS395	*strA1 relA crsF4*	IS56 $\xrightarrow{td}$ CS375
CS410	*strA1 relA crsA47 trpC2*	IS56 $\xrightarrow{td}$ CS377

BGSC: *Bacillus* Genetic Stock Center of the Ohio State University.

$\xrightarrow{tf}$, transformation; $\xrightarrow{td}$, transduction mediated by phage PBS1. Arrows point from the donor to the recipient.

Schaeffer's Sporulation (SP) agar at 4°C. Asporogenous strains were kept frozen at -40°C in Difco Penassay Broth (PA) containing 15% glycerol.

3. Media

(1). Difco Penassay Broth (PA)

(2). Difco Tryptose Blood Agar Base (TBB)

(3). Spizizen's Minimal Medium (MM) (Spizizen 1958)

$(NH_4)_2SO_4$	2.0 g
K_2HPO_4	14.0 g
KH_2PO_4	6.0 g
Sodium citrate	1.0 g
$MgSO_4.7H_2O$	0.2 g
Distilled water	1.0 liter

The pH of this medium was adjusted to 7.0. After autoclaving, 50 mL of 10% glucose was added. MM agar contained 15 g of Bacto-agar (Difco) per liter. For the growth of auxotrophic strains, the medium was supplemented with appropriate amino acids (20 µg/mL), purines (50 µg/mL) or pyrimidines (50 µg/mL).

(4). Schaeffer's Sporulation Medium (SP) (Schaeffer et al. 1965)

Nutrient Broth (Difco)	8.0 g
KCl	1.0 g
$MgSO_4.7H_2O$	0.25 g
$MnCl_2.4H_2O$ (1.9%)	0.1 mL
Distilled water	1.0 liter

The pH of this medium was adjusted to 7.0. After autoclaving, 0.1 mL of $FeSO_4.7H_2O$ (0.27%) and 10 mL of $Ca(NO_3)_2.4H_2O$ (2.3%) were added. SP agar contained 15 g of Bacto-agar (Difco) per liter.

(5). Takahashi's Spore Basal Medium (SBM). (Takahashi 1979)

K_2HPO_4	14.0 g
KH_2PO_4	6.0 g
$MgSO_4.7H_2O$	0.2 g
$MnCl_2.4H_2O$ (1.9%)	0.1 mL
Distilled water	0.5 liter

The pH of this medium was adjusted to 7.0. After autoclaving, the following stock solutions were added prior to use:

Sodium glutamate (10%)	50 mL
$Ca(NO_3)_2.4H_2O$ (2.3%)	10 mL
$FeSO_4.7H_2O$ (0.0027%)	10 mL
Carbon source (0.55M)	100 mL unless otherwise stated

Distilled water final volume: 1 liter

SBM agar contained 15 g of Bacto-agar (Difco) per liter. For the growth of auxotrophic strains, the medium was supplemented with appropriate amino acids (20 µg/mL), purines (50 µg/mL) or pyrimidines (50 µg/mL).

(6). Double-strength Sporulation-Glucose Medium (2xSG) (Leighton and Doi 1971)

Nutrient Broth (Difco)	16.0 g
KCl	2.0 g
$MgSO_4.7H_2O$	0.5 g
$MnCl_2.4H_2O$ (1.9%)	0.1 mL
Distilled water	1.0 liter

The pH was adjusted to 7.0. After autoclaving, the following stock solutions were added:

$Ca(NO_3).4H_2O$ (2.3%)	10 mL
$FeSO_4.7H_2O$ (0.27%)	0.1 mL
Glucose (10%)	10 mL.

4. Bacterial cultures

Liquid cultures were obtained by inoculating cells grown overnight on TBB agar into 10 mL of a medium in a 250-mL flask. To test the effect of catabolites on sporulation, cells were inoculated into SBM containing glucose

or other carbon sources at 55mM. To test the effect of various inhibitors other than catabolites, cells were grown in SP medium and the inhibitory agents were added at half an hour before the end of exponential growth unless otherwise stated. The cultures were grown in a water bath shaker at 37°C for 24 h and the growth was monitored with a Klett-Summerson Colorimeter equipped with a green #54 filter. Various stages of sporulation were designated as follows: t_0, the end of exponential growth; t_1, t_2,..., t_n, hourly interval thereafter. The numbers of viable cells and spores were determined by plating 0.1 mL of appropriately diluted samples on TBB agar and incubated at 37°C for 24 h. To determine the numbers of spores, cultures (1 mL) were heated at 80°C in a water bath for 15 min prior to dilution.

5. Preparation of phage PBS1 lysates

Phage PBS1 lysates used in transduction crosses were prepared and the plaque-forming units were estimated according to Takahashi (1963). Donor strains were grown in PA at 37°C for 4 h and the cultures were diluted 10 times with PA and infected with PBS1 phage stock at a multiplicity of infection of 1. The infected cultures were shaken at 37°C for further 1 h and then placed in an incubator at 37°C for 18 h without shaking. The cell debris were removed by cen-

trifugation (5,000xg, 15 min) and the resulting lysates were sterilized by filtration through a Millipore membrane filter (0.45 um pore size). Strain SB19E was routinely used as indicator bacteria to determine the plaque-forming units of the lysates.

In some cases, a soft agar method was used to prepare the PBS1 lysates. This method was essentially the same as the plaque-assay technique decribed by Takahashi (1963) except that the number of phage particles added was much higher (about $3\text{-}5\times10^5$ per plate). After the plates were incubated overnight at 30°C, 5 mL of PA was added to the agar plates. The soft agar layer was collected in a centrifuge tube and shaken vigorously with a Vortex-Genie mixer. The lysates were obtained by centrifugation and followed by filtration.

6. Preparation of DNA

Procedures for preparing DNA used in transformation crosses were described by Takahashi (1965a). The concentration of DNA was measured by the diphenylamine method (Burton 1956). A standard curve was established with calf thymus DNA solutions.

7. Transduction

Procedures for transduction experiments were as described by Takahashi (1961). Recipient cultures (2 mL) grown in PA for 4 h were mixed with the donor lysate at a multiplicity of infection of about 1. The infected cultures were incubated further with shaking for 30 min and 0.1 mL-samples were plated on appropriate selective agar media.

8. Transformation

Procedures for transformation experiments were according to Takahashi (1965a). Recipient cultures were grown in PA with shaking for 4 h. The cultures were diluted 10 times with MM supplemented with 0.01% Difco Yeast Extract and 0.01% Difco Casamino Acids. The diluted cultures were incubated with shaking for 90 min to establish competent state. To 2 mL of the competent cultures donor DNA was added at final concentrations of 0.02-0.05 µg/mL to determine the frequencies of co-transformation. For the purpose of testing recombination between two unselected markers or introducing an unselected marker into recipient cells, DNA was added at final concentrations of 1-2 µg/mL. The DNA-treated cultures were incubated further with shaking for 30 min and then 0.1 mL-samples were plated on appropriate selective agar media.

9. Selection of recombinants

To select transductants and transformants from recipient strains carrying auxotrophic markers other than dal (D-alanine), the treated cultures were plated on MM agar supplemented with appropriate nutrients and incubated at 37°C for 2-4 days. To select Dal^+ recombinants, samples were plated on TBB plates and incubated for 18 h. To select antibiotic resistant recombinants, 0.1 mL-samples were mixed with 10 mL of melted TBB agar in a petri dish and were incubated for 3 h in order to overcome phenotypic delay. The plates were then overlaid with 10 mL of TBB agar containing each of the following antibiotics: streptomycin (Sigma Chem. Co.), 2 mg/mL; erythromycin (Abbott Laboratories Ltd., Montreal), 5 µg/mL; rifamycin (Sigma Chem. Co.), 100 µg/mL; streptovaricin, 100 µg/mL; streptolydigin, 200 µg/mL. Streptovaricin U-7750 (complex: lot 11560-3) and streptolydigin U-5481 (free acid; lot 2677-Dev-117L) were supplied by Dr. G.B.Whitfield of the Upjohn Co., Kalamazoo, Mich., U.S.A. The plates were incubated for 2 days.

To detect linkage relations between the selected markers and the unselected catabolite resistance (crs) marker, at least 100 transductants or transformants from each cross were streaked twice on the agar plates from which they were selected to obtain single colonies. The colonies

were examined for their morphology. The resistance to glucose was determined in SBM containing glucose at 55mM.

When the linkage relations between the selected marker and other unselected markers were investigated, the recombinants were streaked as patches on appropriate agar media.

10. Identification of relA recombinants

When relA mutation was used as an unselected marker, the relA-carrying recombinants were identified by streaking the colonies on MM agar containing norvaline at 30 µg/mL for crs mutants other than crsA-carrying strains. A concentration of 15 µg/mL was used for crsA mutants. Norvaline interferes with the charging of isoleucine tRNA, resulting in the synthesis of ppGpp in relA$^+$ strains. The ppGpp thus synthesized would positively regulate the synthesis of isoleucine. An elevated concentration of isoleucine would counteract the inhibitory effect of norvaline (Stephens et al. 1975). Since relA mutants are defective in ppGpp synthesis and unable to regulate the isoleucine synthesis, they are more sensitive to norvaline than wild-type strains. Under our experimental condition, relA strains were unable to form colonies on norvaline-containing MM agar due to the lack of stringent response. On the other hand, relA$^+$ strains

formed colonies after 2-4 days of incubation. The results obtained were further confirmed by the amino acid-analogue disk method of Price and Gallant (1982).

11. Mutagenesis and isolation of mutants

For isolation of antibiotic resistant mutants, cells growing exponentially in 10 mL of PA were treated with 0.1 mL of ethylmethanesulfonate (EMS) obtained from Aldrich Chem. Co. Inc. for 20 min. The treated cells were collected by centrifugation and washed twice with PA. The cells were then resuspended in 20 mL of PA and incubated with shaking for 2 h. Mutants that were resistant to rifamycin (50 µg/mL), streptovaricin (50 µg/mL) or streptolydigin (100 µg/mL) were isolated by plating the treated samples on TBB-agar containing respective antibiotic at the concentration indicated.

To determine catabolite resistance of the EMS-induced Rfm^R, Stv^R and Std^R mutants for sporulation, the colonies were replica-plated on SBM containiig glucose at 55mM. After 3 days of incubation at 37°C, colonies showing darker pigmentation were selected and their sporulation in liquid SBM containing glucose was examined.

Suppressor-carrying mutants that were sensitive to glucose for sporulation were isolated from a glucose resistant strain, GLU-47 (crsA47), by the following procedure.

Cells of GLU-47 growing exponentially in 5 mL of PA were treated with 0.05mL of methylmethanesulfonate for 20 min. The treated cells were collected by centrifugation and washed twice with PA. The cells were resuspended in 10 mL of PA and incubated with shaking for 2 h. To enrich the suppressor-carrying Crs^S cells, the cells were collected by centrifugation and resuspended in 10 mL of SBM containing glucose at 55mM. The cultures were incubated for 24 h. Since most GLU-47 cells began the process of sporulation soon after the resuspension, the proportion of mutants that failed to sporulate would gradually increase due to their continuous growth. Then 0.5 mL of the cultures was mixed with 9.5 mL of SP medium and was incubated with shaking for 24 h. The culture (1.0 mL) was heated at 80°C for 15 min to eliminate the possible asporogenous mutants. The heated samples were appropriately diluted and plated on SBM agar containing glucose at 55mM. Colonies which showed light pigmentation on the plates after 3 days of incubation at 37°C were isolated. To confirm the phenotype of the mutant strains isolated, the frequency of spores was determined in liquid SBM containing glucose at 55mM. Suppressor carrying strains that still retained the original crs mutation (crsA47) were identified by transduction and transformation.

12. Resuspension technique

In some experiments, a resuspension technique was used to grow cells, initiate sporulation and to obtain samples for glucose determination and enzyme assay. Cells were grown in SP medium. At $t_{-0.5}$ the cells were collected by centrifugation and resuspended in the same volume of SBM containing glucose or other carbon sources at 55mM.

13. Determination of glucose

Cultures grown in glucose-containing media were chilled in an ice water bath and centrifuged at 5,000xg for 10 min at 4°C. The concentrations of glucose in supernatant fluids were determined by the method of Raabo and Terkildsen (1960) described in Sigma Technical Bulletin No. 510.

14. Preparation of cell extracts

Cell extracts used in the assay of inosine monophosphate (IMP) dehydrogenase and malate dehydrogenase were prepared according to Deutscher and Kornberg (1968). Cultures (20-40 mL) were chilled in an ice water bath and centrifuged for 10 min at 5,000xg in a refrigerated centrifuge. The cells were washed once with buffer A which contained 50mM

Tris-HCl buffer, pH 7.9, 10mM $MgCl_2$, 10mM mercaptoethanol and 0.1mM ethylenediaminetetraacetic acid (Deutscher and Kornberg 1968). The cells were resuspended in 2 mL of Buffer A containing lysozyme (Worthing Biochem. Co. Freehold, N.J., U.S.A.) at 200 µg/mL. After incubation at 37°C for 20 min, the lysate was sonicated with a Braunsonic 1510 for 2 min. Four pulses of 30 sec were used, with 1-min intervals between pulses. Samples were kept in an ice water bath throughout sonication. Essentially no intact vegetative cells could be seen under the phase-contrast microscope after the treatment. The treated samples were centrifuged for 30 min at 17,000xg and the resulting supernatant fluids were used for enzyme assay and protein determination.

15. Enzyme assays

(1). IMP dehydrogenase

IMP dehydrogenase was assayed at 37°C by measuring the rate of increase in the absorbance at 340 nm resulting from the reduction of the oxidized nicotinamide adenine dinucleotide (NAD^+). A Pye Unicam SP30 Spectrophotometer was used to determine the absorbance. The reaction mixture contained 70mM Tris-HCl buffer, pH 8.5, 50mM $(NH_4)_2SO_4$, 25mM glutathione, 8mM NAD^+ and 1mM IMP. The reaction was started by adding 0.1 mL of the cell extract to a pre-warmed

($37^{o}C$) cuvette (light path of 1 cm) containing the reaction mixture. A molar extinction coefficient of $6.23x10^{3}M^{-1}cm^{-1}$ was used to determine the concentration of the reduced form of nicotinamide adenine dinucleotide (NADH). Specific activity was expressed as nanomoles of NADH produced per min per mg of protein.

(2). Malate dehydrogenase

Malate dehydrogenase was assayed at $37^{o}C$ according to the method of Yoshida (1969). The rate of increase in the absorbance at 340 nm resulting from the reduction of NAD^{+} was measured with a Pye Unicam SP30 Spectrophotometer. The reaction mixture contained 50mM Tris-HCl buffer, pH 8.8, 10mM sodium malate and 2mM NAD^{+}. The reaction was started by adding 0.1 mL of the cell extracts to a pre-warmed ($37^{o}C$) cuvette containing the reaction mixture. Specific activity was expressed as nanomoles of NADH produced through the oxidation of malate per min per mg of protein.

(3). Alkaline phosphatase

Alkaline phosphatase was assayed according to the method of Ichikawa and Freese (1974) with the following modifications. Cultures were chilled in an ice water bath and centrifuged at 5,000xg for 10 min. The cells were washed once with 0.1M Tris-HCl buffer, pH 7.6. The cells were re-

suspended in 1.0M Tris-HCl buffer, pH 8.0. One drop of toluene was added into 2.5 mL of the cell resuspension in test tubes. The test tubes were shaken vigorously by hand for 1 min and were kept in a water bath at 37°C. The toluene-treated cell resuspension (0.5 mL) was added into prewarmed test tubes (37°C) containing 1 mL of 1M Tris-HCl buffer, pH 8.8, and 0.5 mL of 10mM nitrophenol phosphate. The mixtures were incubated in a water bath at 37°C for 20 min and the reaction was stopped by adding 1 mL of 2 N NaOH. The mixtures were then centrifuged at 5,000xg for 10 min. Absorbance in the supernatant fluids at 410 nm was measured with a Spectro-20 Spectrophotometer. Specific activity of alkaline phosphatase was expressed as nanomoles of p-nitrophenol produced per min by the amount of cells that gave an absorbance at 600 nm of 1.0 (light path of 1 cm) in 1 mL. A molar extinction coefficient of $16.2 \times 10^{3} M^{-1} cm^{-1}$ was used to determine the concentration of p-nitrophenol.

16. Determination of protein

The concentrations of protein in cell extracts were determined by the method of Lowry et al. (1951). To 0.5 mL of appropriately diluted cell extracts, 2.5 mL of a mixture containing 2% Na_2CO_3 solution in 0.1N NaOH and 0.5% $CuSO_4.5H_2O$ solution in 1% sodium potassium tartrate (50:1) was added and let

stand for 10 min at room temperature. Then 0.25 mL of 1N Folin-Ciocalteau Phenol reagent was added to the above mixture. After 30 min at room temperature, the absorbance at 750 nm was determined with a Spectro-20 Spectrophotometer. A standard curve was established with bovine serum albumin solutions.

17. Electron microscopy

Procedures for electron microscopic examination were described by Takahashi and MacKenzie (1982).

Chapter III

GENETIC MAPPING OF *crs* MUTATIONS

1. Preliminary mapping by PBS1 transduction

To detect linkage relations between *crs* genes and other genes which were already well characterized, a series of transduction experiments were performed with nine Kit strains constructed by Dedonder *et al*. (1977). Genetic markers carried by the Kit strains are linked with each other by transduction mediated by phage PBS1 and cover the entire chromosome of *B. subtilis* (Lepesant-Kejzlarova *et al*. 1975). A mutation can be located on the chromosome by examining its linkage with these reference markers. Since our *crs* mutants are prototrophic and are able to grow on MM containing ammonium sulfate as nitrogen source and glucose as carbon source (Takahashi 1979), it was possible to use these mutants as donor to transduce various auxotrophic markers carried by the Kit strains for prototrophy. The prototrophic transductants were then examined for their ability to sporulate in the presence of inhibitory concentrations of carbon sources.

It was found that mutations *crsA1*, *crsA47*, *crsA4*, *crsC1* and *crsC2* were linked to *aroD120* and *lys1* of Kit-6.

Mutation crsB40 was linked to purB33 of Kit-2. Mutation crsF4 was linked to metC3 carried by Kit-3 and to pyrD1 carried by Kit-4. Strain MAN-1 which was later found to carry two crs mutations showed a linkage with purA16 and cysA14 of Kit-1.

2. Mapping of crsA47, crsA1 and crsA4

Previous studies (Takahashi 1979; Takahashi and MacKenzie 1982) revealed that mutant strains GLU-47 (crsA47), GLU-1 (crsA1) and FRU-4 (crsA4) had very similar phenotypes. These mutants were resistant to glucose not only in sporulation but also in induction of acetoin dehydrogenase (Takahashi 1979). They were also resistant to novobiocin and acridine orange for sporulation (Takahashi and MacKenzie 1982). It is possible therefore that these strains carry mutations which are located within a single locus. This was tested by transformation experiments in which the constructed strains CS1 (trpC2 crsA47), CS4 (trpC2 crsA1) and CS5 (trpC2 crsA4) were transformed for prototrophy with relatively high concentrations of DNA (1.5-2.0 μg/mL) extracted from strains GLU-47, GLU-1, FRU-4 and WS (a wild-type strain). No catabolite sensitive recombinants were found among the Trp^+ transformants (500-3000) obtained in crosses between crsA47, crsA1 and crsA4. On the other hand, approximately 7% of

Trp^+ transformants were catabolite sensitive when strain WS was used as donor. It appears that these three mutations cannot be separated by transformation.

To determine the gene order, three-factor crosses by transduction were carried out. Kit-6 was transduced with lysates of strains GLU-47, GLU-1 and FRU-4 to obtain Aro^+ and Lys^+ transductants. Results presented in Table 2 suggest that the gene order would be "aroD120-(crsA47, crsA1, crsA4)-lys1".

3. Mapping of crsC1 and crsC2

To determine linkage relations between the crsC markers and aroD120 and lys1, Kit-6 was transduced with RIB-2 (crsC1) and GLN-2 (crsC2) as donor. Results of three-factor crosses indicate that both crsC1 and crsC2 are also located between aroD120 and lys1 (Table 3). The co-transduction frequencies for each of the crs markers with aroD120 and lys1 were similar (9-14%). Recombination tests by transformation carried out as in the previous section suggest that the mutations, crsC1 and crsC2, may be in the same locus (data not shown).

Since both crsA and crsC genes were found to be located between aroD120 and lys1, recombination tests between these two genes were carried out. When mutant strain RIB-2

Table 2. Three-factor transduction crosses involving (*crsA47*, *crsA1*, *crsA4*), *aroD120* and *lys1*

Recipient genotype	Donor genotype	Selected marker	Recombinants[a] Aro	Crs[b]	Lys	No. of colonies	Suggested order
aroD120 *lys1* (Kit-6)	*crsA47* (GLU-47)	Aro$^+$	1	0	0	239	*aroD120-crsA47-lys1*
			1	1	0	139	
			1	0	1	0	
			1	1	1	6	
		Lys$^+$	0	0	1	262	
			0	1	1	29	
			1	0	1	1	
			1	1	1	5	
aroD120 *lys1* (Kit-6)	*crsA1* (GLU-1)	Aro$^+$	1	0	0	156	*aroD120-crsA1-lys1*
			1	1	0	100	
			1	0	1	0	
			1	1	1	0	
		Lys$^+$	0	0	1	111	
			0	1	1	4	
			1	0	1	0	
			1	1	1	2	
aroD120 *lys1* (Kit-6)	*crsA4* (FRU-4)	Aro$^+$	1	0	0	115	*aroD120-crsA4-lys1*
			1	1	0	52	
			1	0	1	9	
			1	1	1	0	
		Lys$^+$	0	0	1	102	
			0	1	1	7	
			1	0	1	0	
			1	1	1	1	

[a] 0, recipient phenotype; 1, donor phenotype.

[b] Crs, ability to sporulate in the presence of glucose.

Table 3. Three-factor transduction crosses involving (*crsC1*, *crsC2*), *aroD120* and *lys1*

Recipient genotype	Donor genotype	Selected marker	Recombinants[a] Aro	Crs[b]	Lys	No. of colonies	Suggested order
aroD120 / *lys1* (Kit-6)	*crsC1* (RIB-2)	Aro^+	1	0	0	183	*aroD120-crsC1-lys1*
			1	1	0	18	
			1	0	1	0	
			1	1	1	1	
		Lys^+	0	0	1	266	
			0	1	1	25	
			1	0	1	0	
			1	1	1	2	
aroD120 / *lys1* (Kit-6)	*crsC2* (GLN-2)	Aro^+	1	0	0	373	*aroD120-crsC2-lys1*
			1	1	0	46	
			1	0	1	0	
			1	1	1	6	
		Lys^+	0	0	1	442	
			0	1	1	67	
			1	0	1	1	
			1	1	1	6	

[a] 0, recipient phenotype; 1, donor phenotype.

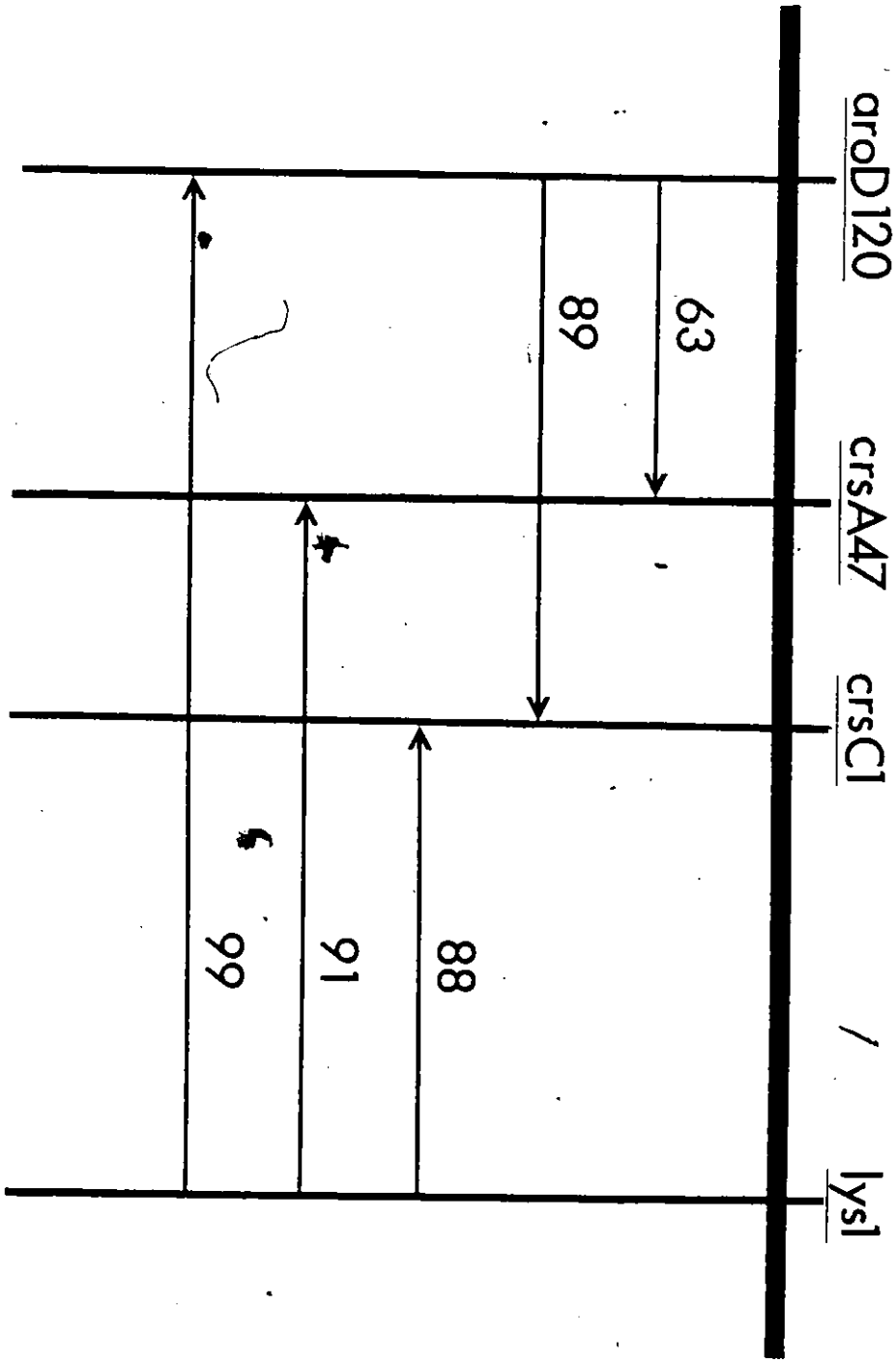
[b] Crs, ability to sporulate in the presence of glucose.

(crsC1) was used as donor to transduce CS2 (trpC2 aroD120 crsA47), 26 of 147 Aro^+ transductants were catabolite sensitive (Crs^S). On the other hand, no Crs^S transductants were found among 56 Lys^+ transductants when CS3 (trpC2 lys1 crsA47), which carried lys1 instead of aroD120, was transduced with lysates of RIB-2. These results would be expected if the gene order were "aroD120-crsA47-crsC1-lys1". In the former cross, the formation of catabolite sensitive recombinants requires only a single crossover, whereas in the latter cross it requires a double crossover. These results indicate that crsA47 and crsC1 can be readily separated by transduction. The suggested gene order would be "aroD120-crsA47-crsC1-lys1" (Fig. 1). Since the map positions of aroD120 and lys1 are 230 and 210, respectively (Henner and Hoch 1980), it is estimated that the map positions of crsA and crsC would be approximately 225 and 220, respectively.

4. Mapping of crsD1 and crsE1

Preliminary results indicated that strain MAN-1 carried a crs mutation which was linked to purA16 and cysA14 by transduction. However, when strain CS19 (purA16 cysA14) was transduced with MAN-1 as donor, three types of Cys^+ transductants were observed. They were arbitrarily designated as type A, B, and C. Type A colony had the phenotype

Fig. 1. Genetic map showing the positions of crsA47 and crsC1. The gene locations were determined by transduction. Arrows point from the selected marker to the unselected marker. Mapping unit distance = 100% - percentage of co-transduction.


aroD120
crsA47
crsC1
lysl
89
63
99
91
88

of Crs^R and appeared smooth on TBB agar. Type B was also Crs^R but was rough on TBB agar. Type C was Crs^S and had the colonial morphology of the recipient strain. When Pur^+ transductants were selected, only type A and C colonies were observed. These observations suggest that MAN-1 carries two crs mutations. Two mutations designated as crsD1 and crsE1 give type A and type B phenotypes, respectively.

When CS21 (cysA14) was transduced for prototrophy with MAN-B1 (crsE1), a type B transductant as donor, approximately 85% of the Cys^+ transductants were type B and 15% were type C, and no type A colonies appeared. When a type A transductant, MAN-A1 (crsD1) was used as donor to transduce CS21, only type A and type C were observed among the Cys^+ transductants. However, when another type A strain, CS24 (crsD1 crsE1) was used as donor, types A, B and C transductants were obtained. These observations suggest that type B strains carry crsE1, whereas type A strains carry either crsD1 alone or both crsD1 and crsE1.

The results of transduction crosses involving crsD1, crsE1, purA16 and cysA14 are shown in Table 4. Since nalA1 (resistance to nalidixic acid), guaA1 and ksgA618 (resistance to kasugamycin) are known to be located between purA16 and cysA14 (see review by Henner and Hoch 1980), they were used as reference markers in the crosses. Results of the above crosses indicate that crsD1 is located between purA16 and

Table 4. Three-factor or four-factor transduction crosses involving *crsD1*, *crsE1* and neighbouring markers

Recipient genotype	Donor genotype	Selected marker	Recombinants[a] Classes[b]			No.	Suggested order
			CrsD	Cys	CrsE		
cysA14 (CS21)	*crsD1* *crsE1* (MAN-1)	Cys^+	0	1	0	7	
			0	1	1	49	
			1	1	0	44	
			1	1	1		
			Pur	CrsD	CrsE		
purA16 (CS20)	*crsD1* *crsE1* (MAN-1)	Pur^+	1	0	0	79	
			1	1	0	21	
			1	1	1		
			1	0	1	0	
			Pur	CrsD	Cys	CrsE	
cysA14 *purA16* (CS19)	*crsD1* *crsE1* (MAN-1)	Cys^+	0	0	1	0	18	*purA16-crsD1-cysA14*
			1	0	1	0	0	
			0	0	1	1	79	
			1	0	1	1	0	
			0	1	1	0	36	
			0	1	1	1		
			1	1	1	0	7	
			1	1	1	1		
		Pur^+	1	0	0	0	68	
			1	0	1	0	0	
			1	0	0	1	0	
			1	0	1	1	0	
			1	1	0	0	15	
			1	1	0	1		
			1	1	1	0	7	
			1	1	1	1		

Table 4. Continued

Recipient genotype	Donor genotype	Selected marker	Recombinants[a] Classes[b]			No.	Suggested order
			CrsD	Cys	CrsE		
crsD1 cysA14 (CS27)	crsE1 (MAN-B1)	Cys$^+$	0	1	0	26	crsD1-cysA14-crsE1
			0	1	1		
			1	1	0	4	
			1	1	1	10	
			Nal	CrsD	Cys		
crsD1 cysA14 (CS27)	nalA1 (WSnal)	Cys$^+$	0	0	1	28	nalA1-crsD1-cysA14
			0	1	1	8	
			1	0	1	0	
			1	1	1	12	
			Pur	Nal	CrsD		
crsD1 purA16 (CS28)	nalA1 (WSnal)	Pur$^+$	1	0	0	31	purA16-nalA1-crsD1
			1	1	0	4	
			1	0	1	1	
			1	1	1	11	
			Gua	CrsD	Cys		
guaA1 ery1 (1A177)	cysA14 crsD1 (CS27)	Gua$^+$	1	0	0	6	guaA1-crsD1-cysA14
			1	1	0	31	
			1	0	1	0	
			1	1	1	16	
			Ksg	CrsD	Cys		
cysA14 crsD1 (CS27)	ksgA618 (1A187)	Cys$^+$	0	0	1	68	ksgA618-crsD1-cysA14
			0	1	1	28	
			1	0	1	3	
			1	1	1	42	

[a] 0, recipient phenotype; 1, donor phenotype.

[b] CrsD, catabolite-resistant transductants which were smooth on TBB agar; CrsE, catabolite-resistant transductants which were rough on TBB agar.

cysA14. Further crosses showed that crsD1 was located at the right of nalA1 (WSnal), guaA1 (1A177) and ksgA618 (1A187). Since the co-transduction frequency of crsE1 with cysA14 (85%) was much higher than that of crsD1 with cysA14 (37%), the location of crsE1 was assumed to be at the right of crsD1. The possibility that crsE1 might be located between crsD1 and cysA14 was eliminated from the observation that type C colonies (Crs^S recombinants) could be readily obtained in crosses between CS27 (crsD1 cysA14) and MAN-B1 (crsE1) in which Cys^+ transductants were selected (Table 4). If the gene order were "crsD1-crsE1-cysA14", it would require double crossover to form type C recombinants. Since transformation crosses to be presented in the next section show that crsE1 is located between cysA14 and strA1, the probable gene order would be "purA16-nalA1-guaA1-ksgA618-crsD1-cysA14-crsE1-strA1" (Fig. 2). Since the map positions of ksgA618, cysA14 and strA1 are 5, 10 and 10, respectively (Henner and Hoch 1980), the map position of crsD1 would be approximately 5 and that of crsE1 would be approximately 10.

5. Further mapping of crsE1

It has been reported that mutations conferring resistance to rifamycin (rfm), streptovaricin (stv) and streptolydigin (std) are located in the structural genes for β

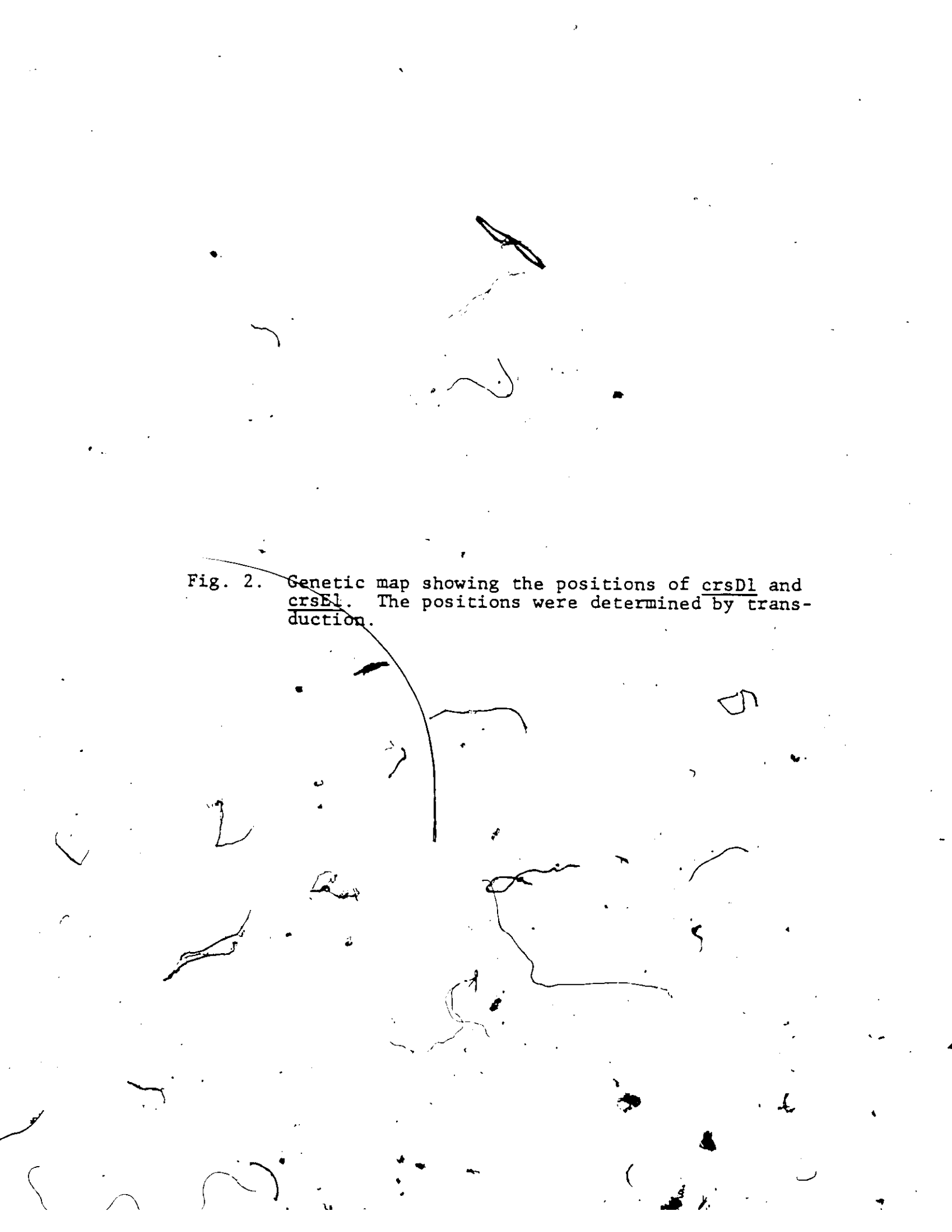
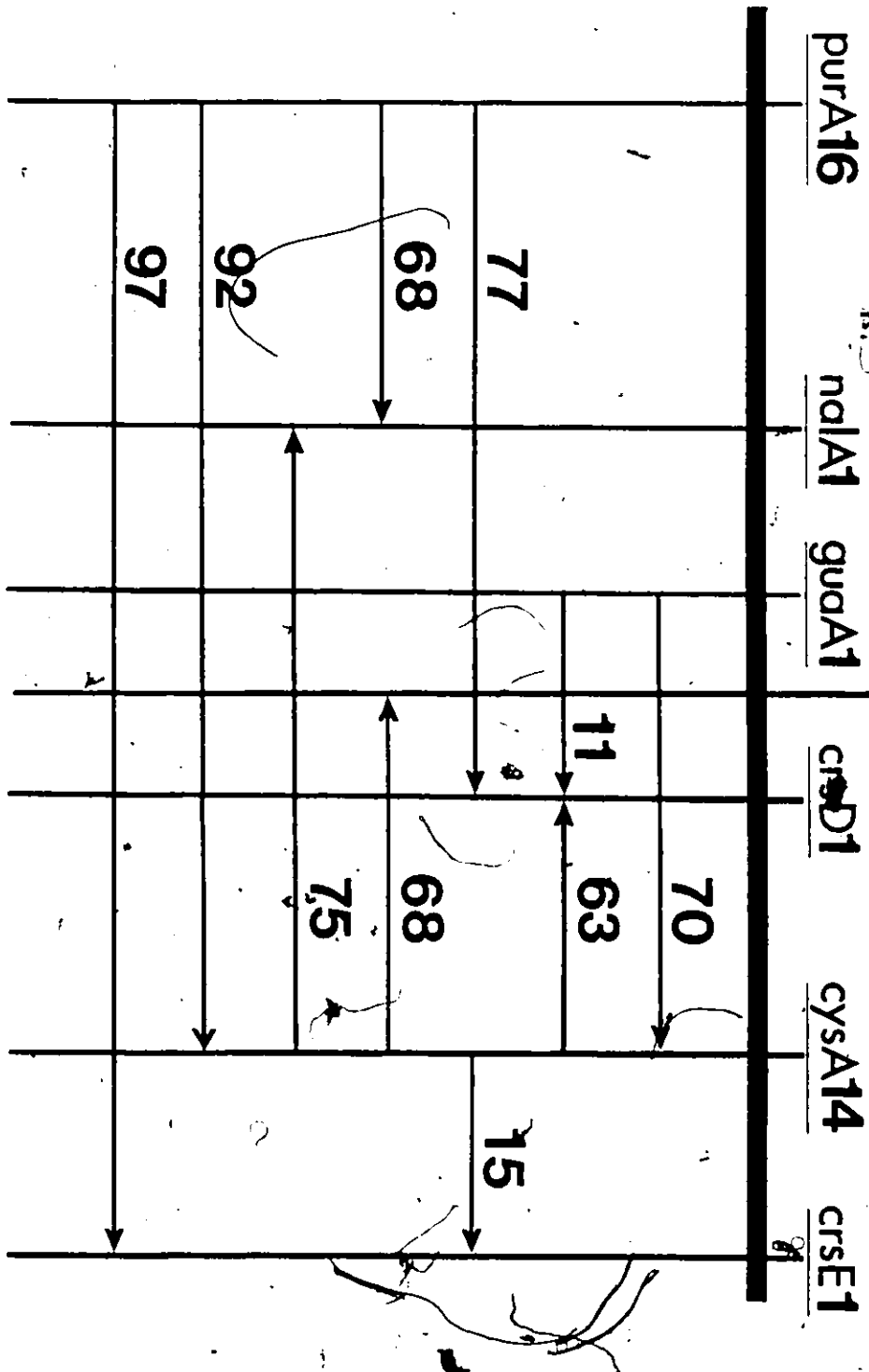


Fig. 2. Genetic map showing the positions of crsD1 and crsE1. The positions were determined by transduction.


purA16
nalA1
guaA1
ksgA618
cysD1
cysA14
crsE1
97
92
68
77
11
75
68
63
70
15

and β' subunits of RNA polymerase (Halling et al. 1977, 1978). These mutations are located between cysA14 and strA1 (Haworth and Brown 1973; Sonenshein et al. 1974). As crsE1 mutation was also located in this region, the sensitivity of MAN-B1 (crsE1) to rifamycin, streptovaricin and streptolydigin was investigated. It was found that vegetative growth of both MAN-B1 and a wild-type strain (CS22) was equally sensitive to these drugs, indicating that the crsE1 mutation is not related to resistance to these drugs. This observation allowed us to use these resistance markers in mapping experiments of the crsE1 mutation. Two rifamycin resistant (Rfm^R) mutants, CS30 (rfm1 crsE1) and CS31 (rfm11 crsE1) were isolated from strain MAN-B1 (crsE1) and used in transformation crosses. Another Rfm^R mutant strain, 1A248 (rfm500) obtained from the Bacillus Genetic Stock Center of Ohio State University, was also included in the experiments. Results shown in Table 5 indicate that crsE1 is located between the rfm mutations and strA1. Co-transformation frequencies of crsE1 with rfm11, rfm500 and rfm1 were 72%, 69% and 60%, respectively.

Since it has been shown that stv and std markers are also located between or close to rfm and strA (Haworth and Brown 1973), these additional markers were also used to map the crsE1 mutation. Two spontaneous Stv^R mutants, CS88 (stv23) and CS90 (stv27) were isolated from a wild-type

Table 5. Three-factor transformation crosses involving *crsE1* and neighbouring markers

Recipient genotype	Donor genotype	Selected marker	Recombinants[a] Classes[b]			No. of colonies	Suggested order
			Cys	crsE	Str		
cysA14 (CS21)	*crsE1 strA1* (CS29)	Cys^+	1	0	0	110	*cysA14-crsE1-strA1*
			1	1	0	21	
			1	0	1	14	
			1	1	1	54	
		Str^R	0	0	1	71	*cysA14-crsE1-strA1*
			0	1	1	139	
			1	0	1	10	
			1	1	1	82	
			Rfm	crsE	Str		
crsE1 rfm1 (CS30)	*strA1* (WS)	Str^R	0	0	1	28	*rfm1-crsE1-strA1*
			0	1	1	16	
			1	0	1	1	
			1	1	1	61	
crsE1 rfm11 (CS31)	*strA1* (WS)	Str^R	0	0	1	24	*rfm11-crsE1-strA1*
			0	1	1	17	
			1	0	1	3	
			1	1	1	61	
strA1 (CS23)	*crsE1 rfm1* (CS300)	Rfm^R	1	0	0	180	*rfm1-crsE1-strA1*
			1	1	0	55	
			1	0	1	7	
			1	1	1	219	
strA1 (CS23)	*crsE1 rfm11* (CS31)	Rfm^R	1	0	0	43	*rfm11-crsE1-strA1*
			1	1	0	15	
			1	0	1	6	
			1	1	1	109	

Table 5. Continued

Recipient genotype	Donor genotype	Selected marker	Recombinants[a] Classes[b]			No. of colonies	Suggested order
			Rfm	crsE	Str		
strA1 crsE1 (CS29)	rfm500 (1A248)	Rfm^R	1	0	0	64	rfm500-crsE1-strA1
			1	1	0	38	
			1	0	1	4	
			1	1	1	113	

[a]0, recipient phenotype; 1, donor phenotype.

[b]CrsE, catabolite-resistant transformants which were rough on TBB agar.

strain CS22, and an Std^R mutant, CS75 (std4 crsE1) was isolated from MAN-B1 (crsE1). These resistant strains were used in three-factor transformation crosses. Results presented in Table 6 indicate that crsE1 is located between std4 and stv mutations. Co-transformation frequency between crsE1 and std4 was 81%. From the above results, it is suggested that the gene order would be "rfm1-rfm500-(rfm11, stv23-stv27)-crsE1-std4-strA1" (Fig. 3).

6. Mapping of crsB40

A glucose resistant mutant, GLU-40 which requires high concentrations of glucose for sporulation, has properties that are distinct from those of GLU-1 and GLU-47 (Takahashi 1979; Takahashi and MacKenzie 1982). Preliminary experiments indicated that the mutation crsB40 was linked to purB33. The frequency of co-transduction and that of co-transformation between crsB40 and purB33 were 85% and 38% respectively. From the map position of purB33, which is 55 (Henner and Hoch 1980), the map position of crsB40 is assumed to be approximately 55. No linkage was observed between crsB40 and other auxotrophic markers tested.

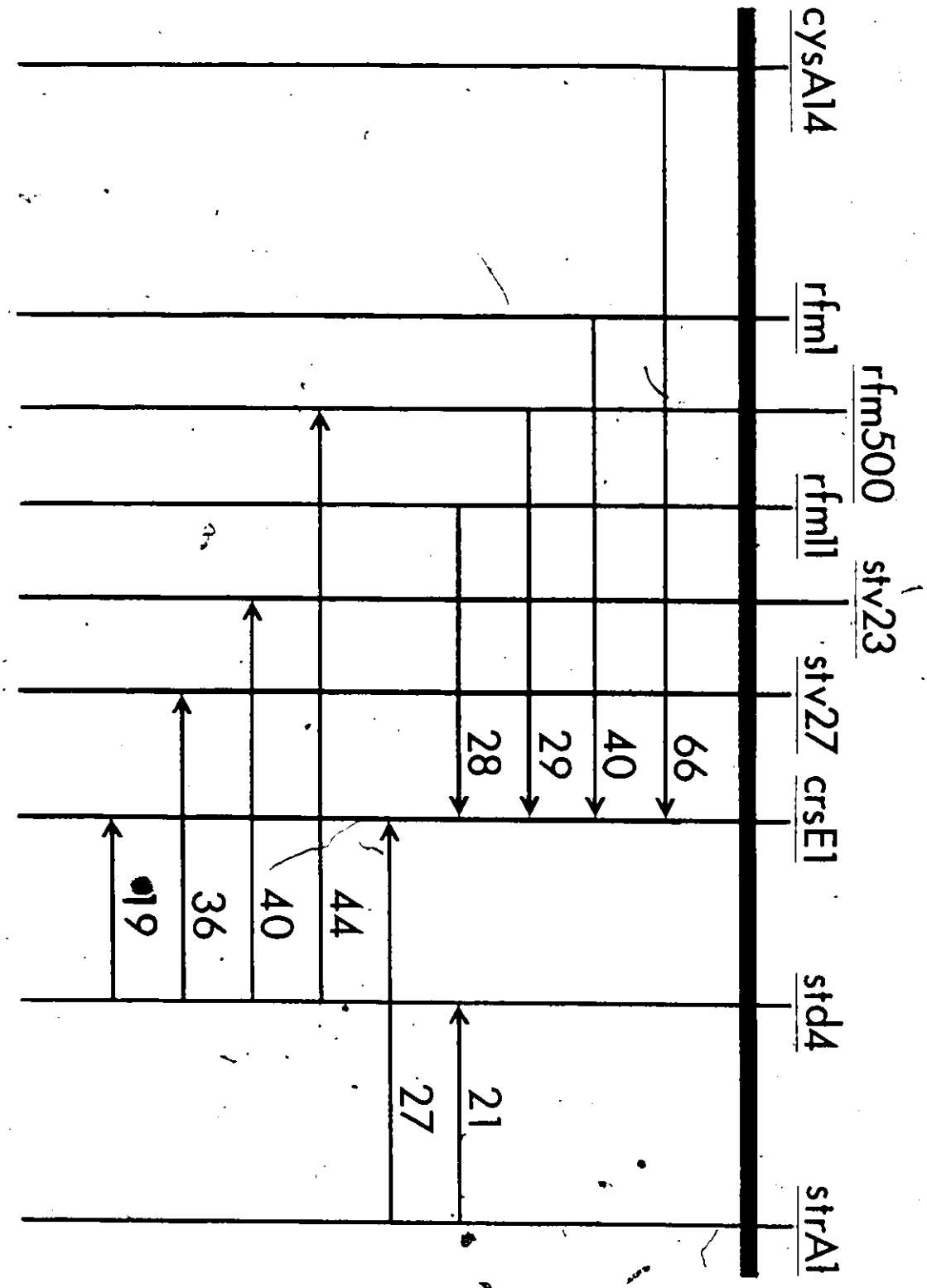
Table 6. Three-factor transformation crosses involving *crsE1* and neighbouring markers

Recipient genotype	Donor genotype	Selected marker	Recombinants[a] Classes[b]			No. of colonies	Suggested order
			Rfm	CrsE	Std		
std4 *crsE1* (CS75)	*rfm500* (1A248)	Rfm^R	1	0	0	127	*rfm500-crsE1-std4*
			1	1	0	88	
			1	0	1	9	
			1	1	1	283	
rfm500 (1A248)	*std4* *crsE1* (CS75)	Std^R	0	0	1	89	*rfm500-crsE1-std4*
			0	1	1	123	
			1	0	1	7	
			1	1	1	261	
			CrsE	Std	Str		
std4 *crsE1* (CS75)	*strA1* (WS)	Str^R	0	0	1	28	*crsE1-std4-strA1*
			0	1	1	16	
			1	0	1	7	
			1	1	1	114	
			Stv	CrsE	Std		
stv23 (CS88)	*std4* *crsE1* (CS75)	Std^R	0	0	1	35	*stv23-crsE1-std4*
			0	1	1	51	
			1	0	1	6	
			1	1	1	124	
stv27 (CS90)	*std4* *crsE1* (CS75)	Std^R	0	0	1	26	*stv27-crsE1-std4*
			0	1	1	31	
			1	0	1	3	
			1	1	1	98	

[a] 0, recipient phenotype; 1, donor phenotype.

[b] CrsE, catabolite-resistant transformants which were rough on TBB agar.

Fig. 3. Genetic map showing the relative position of crsE1. The gene locations were determined by transformation. Mapping unit distance = 100% - percentage of co-transformation.


cysA14
rfm1
rfm500
rfm11
stv23
stv27
crsE1
std4
strA1
66
40
29
28
44
40
36
19
27
21

7. Mapping of crsF4

Preliminary results indicated that crsF4 mutation carried by MAL-4 was linked to metC3 (Kit-3) and pyrD1 (Kit-4) by transduction. To determine the location of crsF4, an asporogenous marker (spo0E11) which is located between metC3 and pyrD1 (quoted in Piggot and Coote 1976), was used as a reference market. When CS71 (metC3 crsF4) was transduced with strain 1S17 (spo0E11) as donor, it was found that, of 461 Met^+ transductants examined 255 were Spo^+ and Crs^R, 200 were Spo^-, and only 6 were Spo^+ and Crs^S (Table 7). If the gene order were "crsF4-metC3-spo0E11", the catabolite resistant recombinants would be produced at a very low frequency, since the formation of this type of transductants requires double crossover. In fact, the catabolite resistant Spo^+ transductants were more abundant than other classes of transductants. This observation therefore eliminated the gene order, "crsF4-metC3-spo0E11". Transformation crosses revealed that crsF4 and spo0E11 could be readily separated (data not shown). Since the phenotypes of crsF4 spo0E11 double mutant strain and the spo0E11 strain were not distinguishable, the relative positions of crsF4 and spo0E11 could not be established from the present data. From the map position of spo0E11, crsF4 is assumed to be located at a map position of approximately 120.

Table 7. Transduction crosses involving crsF4, metC3, pyrD1, and spo0E11

Recipient genotype	Donor Genotype	Selected marker	Recombinants[a] Classes[b]			No. of colonies	Suggested order
			Met	CrsF			
metC3 (CS13)	crsF4 (MAL-4)	Met^+	1	0		205	
			1	1		104	
				CrsF	Pyr		
pyrD1 (CS14)	crsF4 (MAL-4)	Pyr^+		0	1	445	
				1	1	5	
			Met	CrsF	Spo		
metC3 crsF4 (CS71)	spo0E11 (1S17)	Met^+	1	0	0	255	metC3-crsF4-spo0E11
			1	1	0	6	
			1	0	1	200	
			1	1	1		

[a] 0, recipient phenotype; 1, donor phenotype.

[b] CrsF, catabolite-resistant transductants showing MAL-4 phenotype; Spo, asporogenous transductants which were recognized by the absence of pigment on MM agar supplemented with $MnCl_2$ at 1.9 mg/L.

Chapter IV

CHARACTERIZATION OF *crs* MUTANTS

1. Growth, sporulation and glucose utilization of GLU-47

It was reported that *B. subtilis* mutants which were defective in glucose transport (Freese *et al.* 1970) or malate metabolism (Ohné and Rutberg 1976) sporulated well in the presence of these compounds. To determine whether the Crs^R phenotype of strain GLU-47 is due to a similar defect, growth rate and glucose utilization of this mutant were investigated. Cells were grown in SBM containing glucose. The rate of growth as expressed in doubling time was determined during the exponential growth phase. The doubling time of GLU-47 was 148 min, which was much longer than that of a wild-type strain, WS (50 min) under the same condition (Table 8).

The resuspension technique described in Chapter II was used to determine glucose utilization and spore formation. It was found that the frequency of spores in GLU-47 was 0.67, 7 h after cells were resuspended in SBM containing glucose (Fig. 4). This indicates that a majority of cells have started sporulation soon after resuspension. In contrast, the frequency of spores in WS was less than 10^{-4} even

Tanle 8. Growth rates of strains GLU-47 and WS in SBM containing different carbon sources

Carbon source	Doubling time (min)	
	GLU-47	WS
Glucose	148	50
Fructose	148	50
Malate	154	76
Glycerol	156	58
Ribose	184	70
Gluconate	188	109

Note: Cells were inoculated into SBM containing various carbon sources at 55mM. The doubling times were determined during the exponential growth phase.

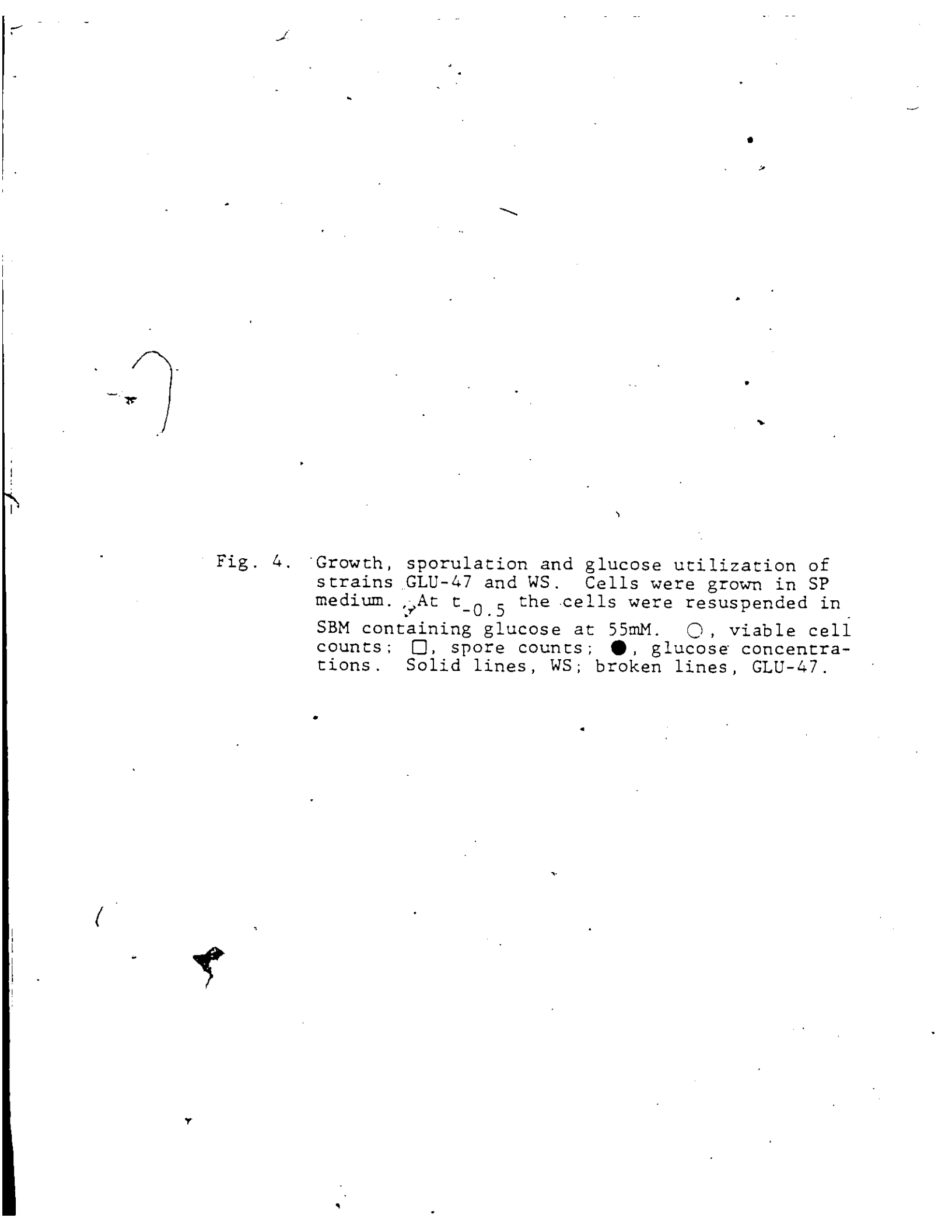
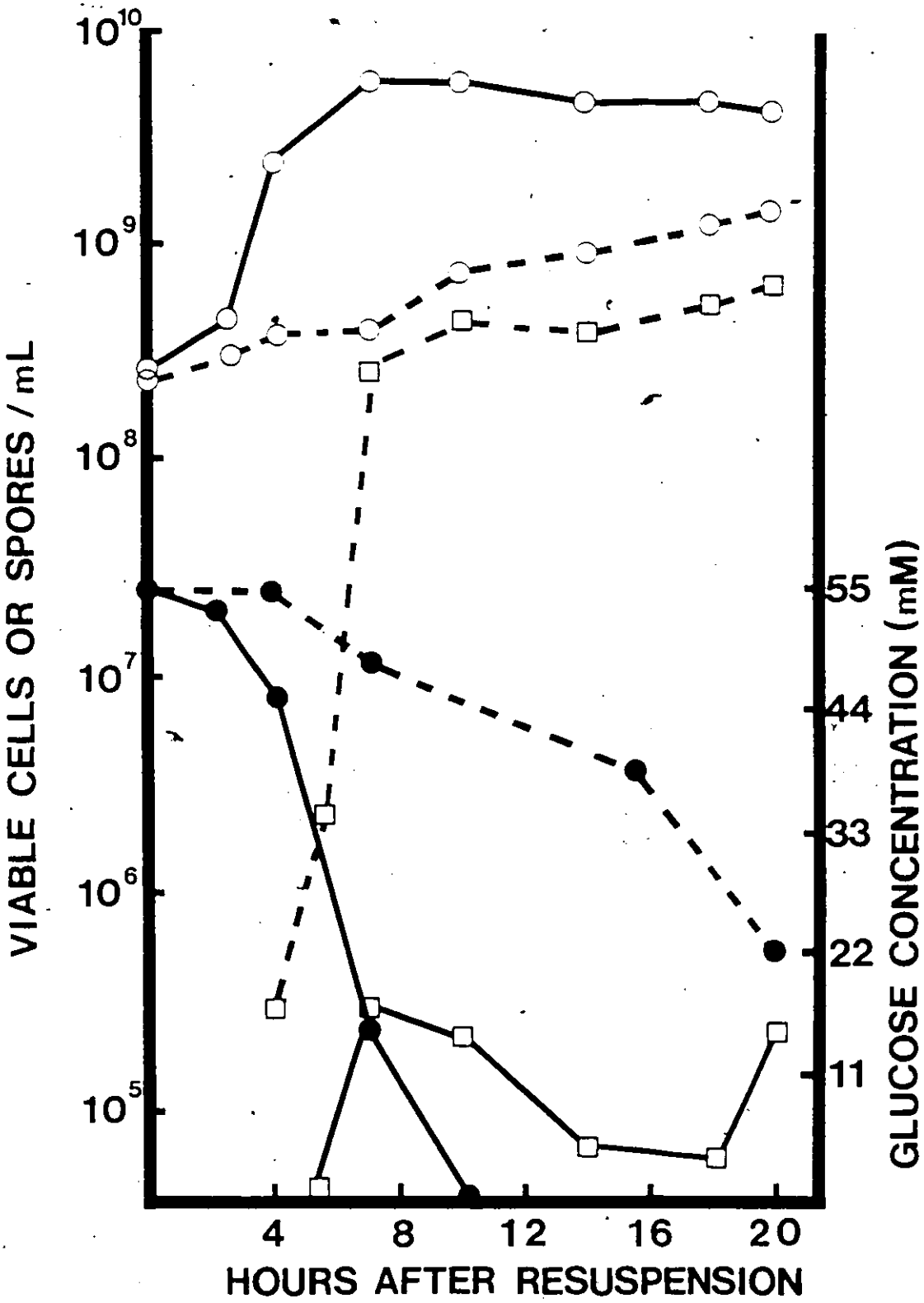


Fig. 4. Growth, sporulation and glucose utilization of strains GLU-47 and WS. Cells were grown in SP medium. At $t_{-0.5}$ the cells were resuspended in SBM containing glucose at 55mM. ○, viable cell counts; □, spore counts; ●, glucose concentrations. Solid lines, WS; broken lines, GLU-47.


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
VIABLE CELLS OR SPORES / mL
55
44
33
22
11
GLUCOSE CONCENTRATION (mM)
4
8
12
16
20
HOURS AFTER RESUSPENSION

20 h after resuspension. Glucose was almost completely utilized in the wild-type culture 10 h after the resuspension. In GLU-47, on the other hand, only 60% of glucose was metabolized after 20 h (Fig. 4).

To determine whether the slow growth of GLU-47 and its slow utilization of glucose were due to a deficiency in glucose transport or metabolism, the growth rates of GLU-47 and WS in SBM containing carbon sources other than glucose were determined. It was found that the growth rate of GLU-47 was again slower than that of WS with all carbon sources tested (Table 8). The doubling times of GLU-47 with these carbon sources varied from 148 min to 188 min, while those of WS varied from 50 min to 109 min (Table 8). Since cells take up and utilize these carbon sources through different mechanisms, the results seem to indicate that the slow rates of growth and glucose utilization in GLU-47 are due to altered cellular functions other than glucose transport and metabolism.

2. Growth, sporulation and glucose utilization of other *crs* mutants

The growth rate of other *crs* mutant strains in SBM containing glucose was also investigated. It was found that the doubling time of strain MAL-4 (*crsF4*) was similar

to that of GLU-47 (crsA47) (Table 9). On the other hand, the doubling times of strains RIB-2 (crsC1), MAN-A1 (crsD1) and MAN-B1 (crsE1) were similar to or only slightly longer than that of the wild-type strain (WS) (Table 9). Glucose utilization and spore formation in the above crs mutants were determined by the resuspension technique. Glucose was almost completely metabolized by RIB-2, GLN-2 and MAN-A1 after 10 h of incubation (Table 10). During the same period, both MAL-4 and MAN-B1 metabolized about 70-80% of glucose (Table 10). Strains GLU-47 and MAL-4 sporulated well after 10 h of incubation, while other crs mutant strains did so after 20 h. The above results indicate that although the crs mutants show the same Crs^R phenotype, their growth characteristics and the rates of glucose utilization vary considerably.

3. Changes in enzymatic activities in crs mutants

(1). IMP dehydrogenase

Deutscher and Kornberg (1968) reported that the level of IMP dehydrogenase decreased at the beginning of sporulation. Freese et al. (1979b) found that the addition of mycophenolic acid, an inhibitor of IMP dehydrogenase could initiate sporulation in the presence of excess glucose, ammonia and phosphate. To determine the relation between

Table 9. Growth rates of *crs* mutants in SBM containing glucose at 55mM

Strain	Doubling time (min)
GLU-47 (*crsA47*)	148
RIB-2 (*crsC1*)	61
MAN-A1 (*crsD1*)	55
MAN-B1 (*crsE1*)	73
MAL-4 (*crsF4*)	120
WS (wild-type)	50

Note: Cells were inoculated into SBM containing glucose at 55mM. The doubling times were determined during the exponential growth phase.

Table 10. Glucose utilization and sporulation of *crs* mutants

Strain	Glucose utilized (%)		Frequency of spores	
	10 h	20 h	10 h	20 h
GLU-47 (*crsA47*)	13	60	5.7×10^{-1}	5.6×10^{-1}
RIB-2 (*crsC1*)	100	100	5.5×10^{-3}	1.0×10^{-1}
GLN-2 (*crsC2*)	98	100	5.5×10^{-3}	5.0×10^{-1}
MAN-A1 (*crsD1*)	100	100	2.0×10^{-3}	5.0×10^{-1}
MAN-B1 (*crsE1*)	80	100	1.6×10^{-2}	2.0×10^{-1}
MAL-4 (*crsF4*)	69	100	1.1×10^{-1}	4.4×10^{-1}
WS (wild-type)	100	100	4.7×10^{-5}	6.4×10^{-5}

Note: Cells were grown in SP medium. At $t_{-0.5}$ the cells were resuspended in the same volume of SBM containing glucose at 55mM. The viable cell counts varied from 8.4×10^{8}/mL to 4.7×10^{9}/mL.

IMP dehydrogenase and catabolite resistance in sporulation, the levels of this enzyme in various crs mutants were determined. At the time of resuspension, specific activity of IMP dehydrogenase in strain GLU-47 (crsA47) was only about 30% of that in WS (Fig. 5). After the resuspension, the level of this enzyme in GLU-47 decreased considerably regardless of the presence or absence of glucose. A sharp decrease in the level of IMP dehydrogenase was observed in WS cells resuspended in SBM without glucose, a condition under which WS cells sporulated well after 8 h. In the presence of glucose, however, a considerable amount of enzyme activity was still present in WS cells 7 h after the resuspension (Fig. 5).

Specific activities of IMP dehydrogenase found in cells of other crs mutants are summarized in Table 11. At the time of resuspension, specific activities of this enzyme in strains MAN-B1, MAN-A1, GLN-2, RIB-2 and MAL-4 were 45-80% of that in WS. After 4h of incubation in SBM containing glucose, the enzyme activities in MAN-B1 and MAL-4 decreased considerably as in the case of GLU-47. On the other hand, the levels of this enzyme in RIB-2, GLN-2 and MAN-A1 decreased only slightly (Table 11). It is suggested that this enzyme may not be closely related to the Crs^R phenotype of crs mutants.

Fig. 5. Specific activity of IMP dehydrogenase in strains GLU-47 and WS. Cells were grown in SP medium. At $t_{-0.5}$ the cells were resuspended in SBM containing glucose at 55mM (■) or no glucose (●). Solid lines, WS; broken lines, GLU-47.

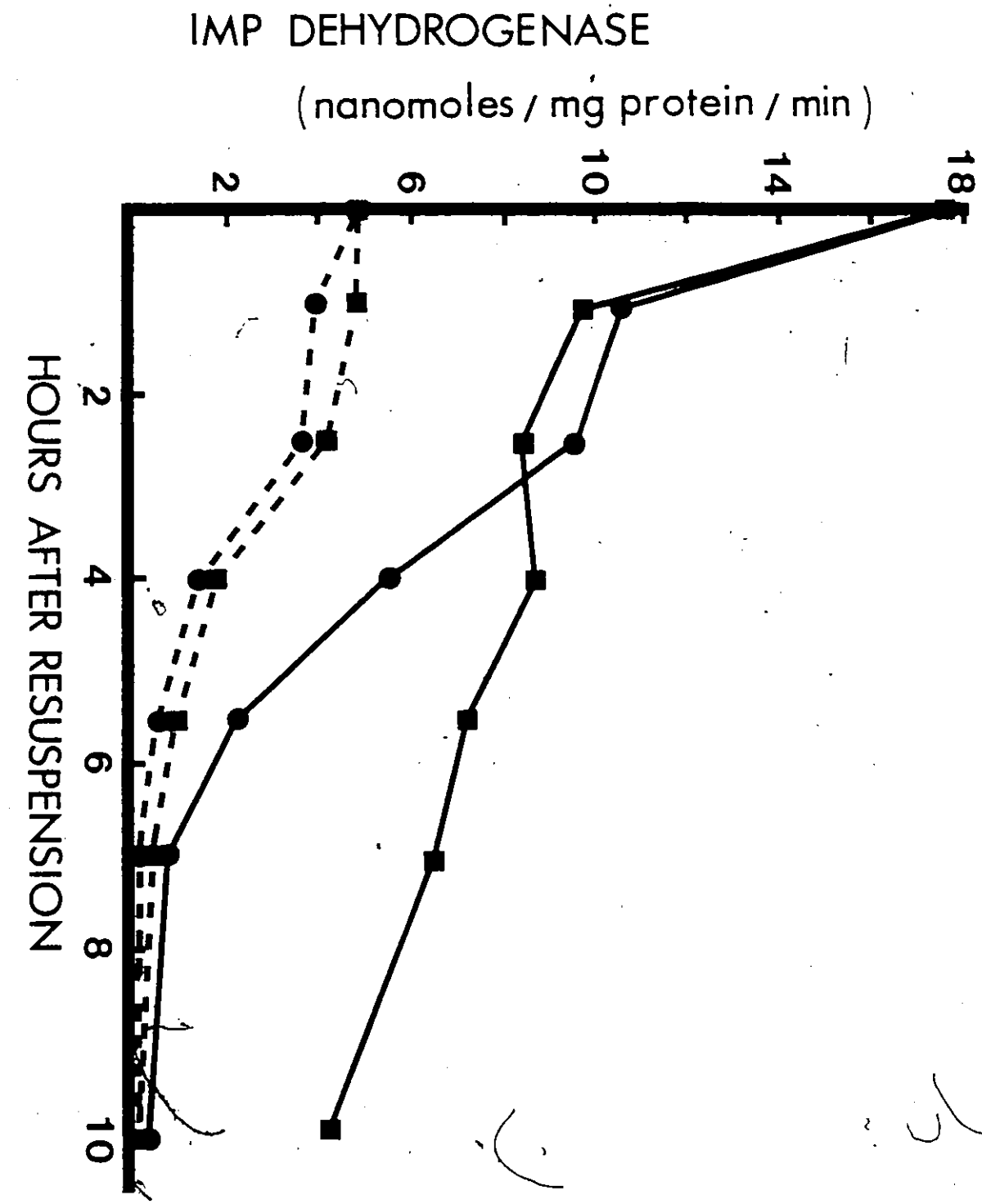

IMP DEHYDROGENASE
(nanomoles / mg protein / min)
2
6
10
14
18
HOURS AFTER RESUSPENSION
2
4
6
8
10

Table 11. Specific activity of IMP dehydrogenase in crs mutants

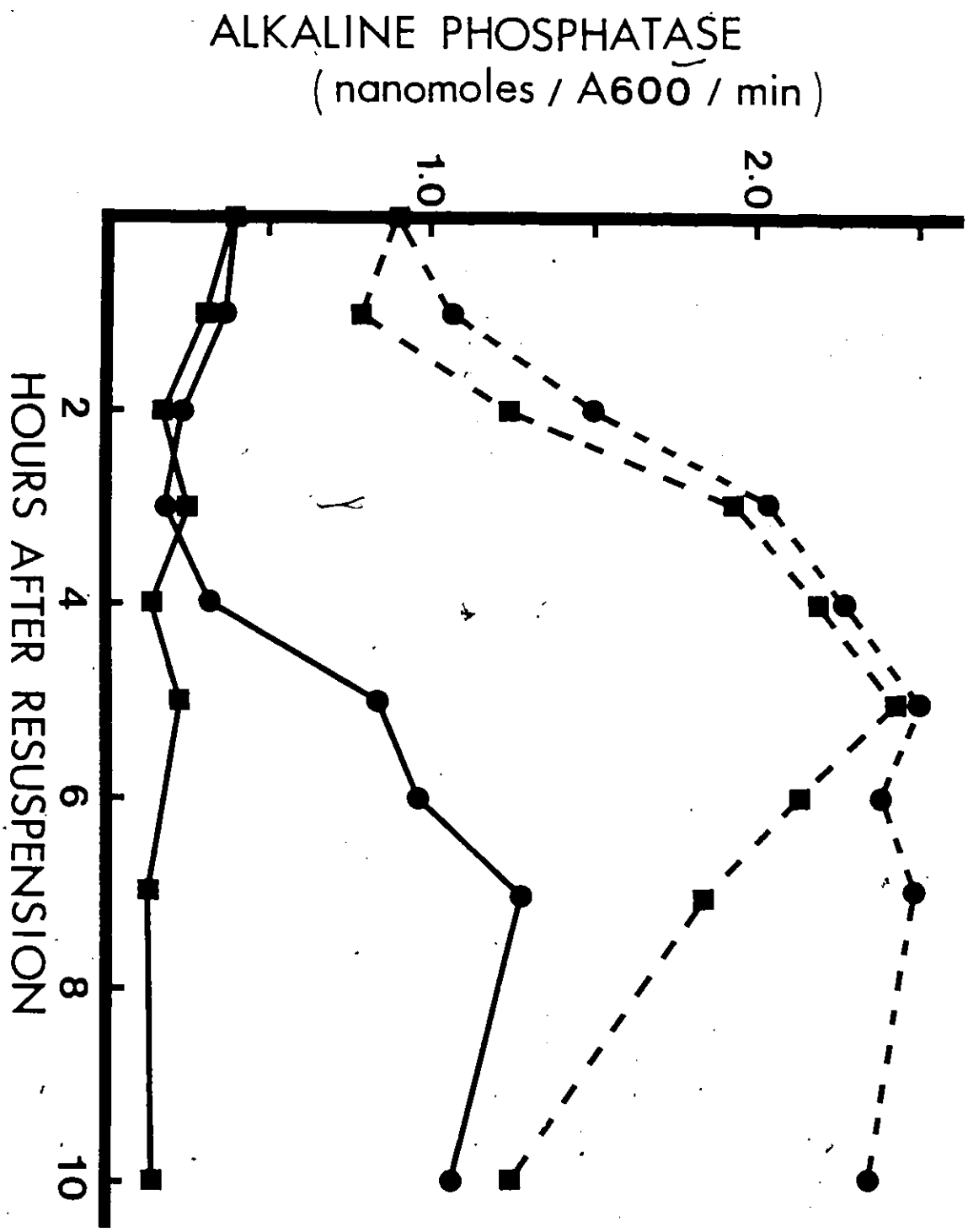
Strain	Specific activity of IMP dehydrogenase	
	0 h	4 h
GLU-47 (crsA47)	5.3	1.7
RIB-2 (crsC1)	11.3	11.0
GLN-2 (crsC2)	11.0	9.9
MAN-A1 (crsD1)	10.2	8.3
MAN-B1 (crsE1)	8.2	3.0
MAL-4 (crsF4)	14.4	3.5
WS (with glucose)	17.9	9.2
WS (without glucose)	17.9	5.8

Note: Cells were grown in SP medium. At $t_{-0.5}$ the cells were resuspended in the same volume of SBM containing glucose at 55mM. Specific activity of IMP dehydrogenase was assayed at the time of resuspension (0 h) and at 4 h after it.

(2). Alkaline phosphatase

Waites et al. (1970) reported that specific activity of alkaline phosphatase increased significantly during stage II of sporulation. The synthesis of this enzyme could be repressed by inorganic phosphate during vegetative growth but not during sporulation (Glenn and Mandelstam 1971; Ichikawa and Freese 1974). Although the function of alkaline phosphatase during sporulation is not clear, the synthesis of this enzyme is considered to be one of the marker events that are associated with sporulation (Young and Mandelstam 1979). The levels of alkaline phosphatase in crs mutants were determined by the following technique. Cells were grown in SP medium supplemented with phosphate buffer (10mM), pH7.0, to repress the synthesis of alkaline phosphatase during vegetative growth. At $t_{-0.5}$ the cells were resuspended in SBM with or without the addition of glucose. In contrast to the case of IMP dehydrogenase, it was found that the background level of alkaline phosphatase of GLU-47 was much higher than that of WS at the time of cell resuspension (Fig. 6). In SBM containing glucose, specific activity of alkaline phosphatase in WS remained at a very low level throughout the experiment. In the absence of glucose, a significant increase in the enzyme activity in WS was observed after 4 h of incubation. This increase was expected since WS cells sporulated well in SBM without

Fig. 6. Specific activity of alkaline phosphatase in strains GLU-47 and WS. Cells were grown in SP medium supplemented with phosphate buffer (10mM), pH7.0. At $t_{-0.5}$ the cells were resuspended in SBM containing glucose at 55mM (■) or no glucose (●). Solid lines, WS; broken lines, GLU-47.


ALKALINE PHOSPHATASE
(nanomoles / A600 / min)
1.0
2.0
HOURS AFTER RESUSPENSION
2
4
6
8
10

glucose. In GLU-47, an increase in the enzyme activity occured 1 h after cell resuspension regardless of the presence or absence of glucose. Furthermore, the highest enzyme activity observed in GLU-47 was twice that in WS without glucose (Fig. 6).

The effect of glucose on alkaline phosphatase in other crs mutants was also investigated. Specific activities of this enzyme in strains RIB-2, GLN-2, MAN-A1, MAN-B1 and MAL-4 varied from 40% to 170% of that in WS at the time of cell resuspension (Table 12). After 4 or 5.5 h of incubation in SBM without glucose, the level of this enzyme increased in all the strains tested. This is expected since all the strains sporulated well after 10 h under this condition. In the presence of glucose, the increase was observed only in GLU-47, MAN-B1 and MAL-4. The enzyme activities in RIB-2 and GLN-2 remained at a low level. In contrast, MAN-A1 showed a decrease in the enzyme activity (Table 12). These results indicate that glucose suppresses the increase in alkaline phosphatase activity in strains RIB-2, GLN-2 and MAN-A1 but not in GLU-47, MAN-B1 and MAL-4. Therefore, the effect of glucose on alkaline phosphatase in crs mutants seems to be variable. It may be concluded that the level of this enzyme is not directly related to Crs^R phenotype of these mutants.

Table 12. Effect of glucose on specific activity of alkaline phosphatase in crs mutants

Strain	Addition of glucose	Alkaline phosphatase		
		0 h	4 h	5.5 h
GLU-47	+	0.91	2.25	2.33
(crsA47)	-	0.91	2.31	2.48
RIB-2	+	0.15	ND	0.10
(crsC1)	-	0.15	ND	1.78
GLN-2	+	0.25	ND	0.13
(crsC2)	-	0.25	ND	1.43
MAN-A1	+	0.43	ND	0.07
(crsD1)	-	0.43	ND	1.85
MAN-B1	+	0.43	0.71	ND
(crsE1)	-	0.43	1.39	ND
MAL-4	+	0.62	1.04	ND
(crsF4)	-	0.62	1.33	ND
WS	+	0.36	0.16	0.20
(wild-type)	-	0.36	0.31	0.85

Note: Cells were grown in SP medium supplemented with phosphate buffer (10mM), pH7.0. At $t_{-0.5}$ the cells were resuspended in the same volume of SBM containing glucose at 55mM (+) or without glucose (-). ND: not determined.

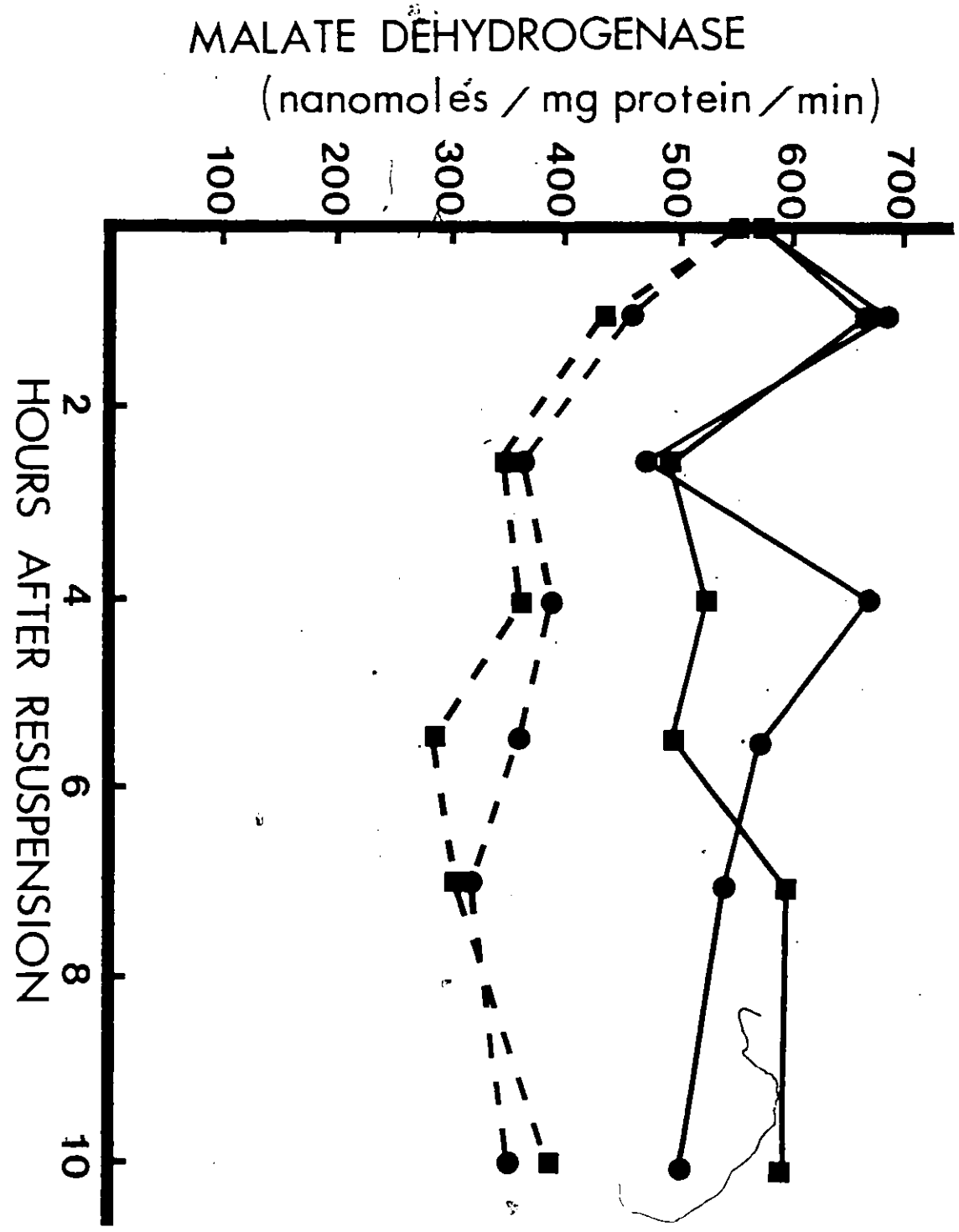
(3). Malate dehydrogenase

It has been known that the activity of malate dehydrogenase undergoes a significant increase during the initial stages of sporulation (Hanson et al. 1963; Deutscher and Kornberg 1968). To determine whether the level of malate dehydrogenase is related to Crs^R phenotype, this enzyme was assayed in strains GLU-47 and WS. Cells were grown in SP medium and at $t_{-0.5}$ the cells were resuspended in SBM with or without the addition of glucose. At the time of cell resuspension, specific activities of malate dehydrogenase in GLU-47 and WS were similar (Fig. 7). After the resuspension, no significant change in the enzyme activity was found in WS up to 10 h. In GLU-47, the enzyme activity decreased to about 60% of its original level 2.5 h after resuspension and thereafter remained at a relatively constant level (Fig. 7). Since the presence or absence of glucose did not significantly influence the level of malate dehydrogenase in both GLU-47 and WS (Fig. 7), this enzyme may not be closely related to the inhibitory effect of glucose on sporulation and to the Crs^R phenotype of the mutant strain.

4. Effect of various inhibitors on sporulation of crs mutants

It was reported that a variety of agents such as

Fig. 7. Specific activity of malate dehydrogenase in strains GLU-47 and WS. Cells were grown in SP medium. At $t_{-0.5}$ the cells were resuspended in SBM containing glucose at 55mM (■) or no glucose (●). Solid lines, WS; broken lines, GLU-47.


MALATE DEHYDROGENASE
(nanomoles / mg protein / min)
100
200
300
400
500
600
700
2
4
6
8
10
HOURS AFTER RESUSPENSION

novobiocin (Takahashi and MacKenzie 1982), cerulenin (Wayne et al. 1981) and aliphatic alcohols (Bohin et al. 1976b) could inhibit sporulation of wild-type strains of B. subtilis. Bohin and Lubochinsky (1982) succeeded in isolating a number of ssa mutants that were able to sporulate in the presence of aliphatic alcohols. Takahashi and MacKenzie (1982) observed that certain crs mutants were resistant to novobiocin in sporulation.

Wayne et al. (1981) reported that low concentrations (1-4 µg/mL) of cerulenin added at the time of inoculation greatly reduced sporulation of wild-type cells at 47°C but not at 37°C: temperature sensitive asporogenous phenotype (Spo^{ts}). However, it was found that under our condition cerulenin could inhibit sporulation of a wild-type strain (WS) even at 37°C. Cerulenin added to the culture at the time of inoculation or at $t_{-0.5}$ greatly reduced the spore yield in the wild-type culture (Fig. 8). The discrepancy between our results and those of Wayne et al. (1981) may be due to the different culture media used in the experiments.

To determine the stage at which sporulation is blocked by cerulenin, samples were taken at different times from a wild-type culture (WS) to which cerulenin (1 µg/mL) was added at $t_{-0.5}$ and the percentage of cells showing different stages of sporulation was determined under the electron microscope. As shown in Table 13, the great majority

Fig. 8. Effect of cerulenin on sporulation of a wild-type strain (WS). Cells were grown in SP medium. Cerulenin at various concentrations was added at the time of inoculation (□) or at $t_{-0.5}$ (○). In all experiments, the viable cell count per mL varied from 3.5×10^8 to 6.3×10^8.

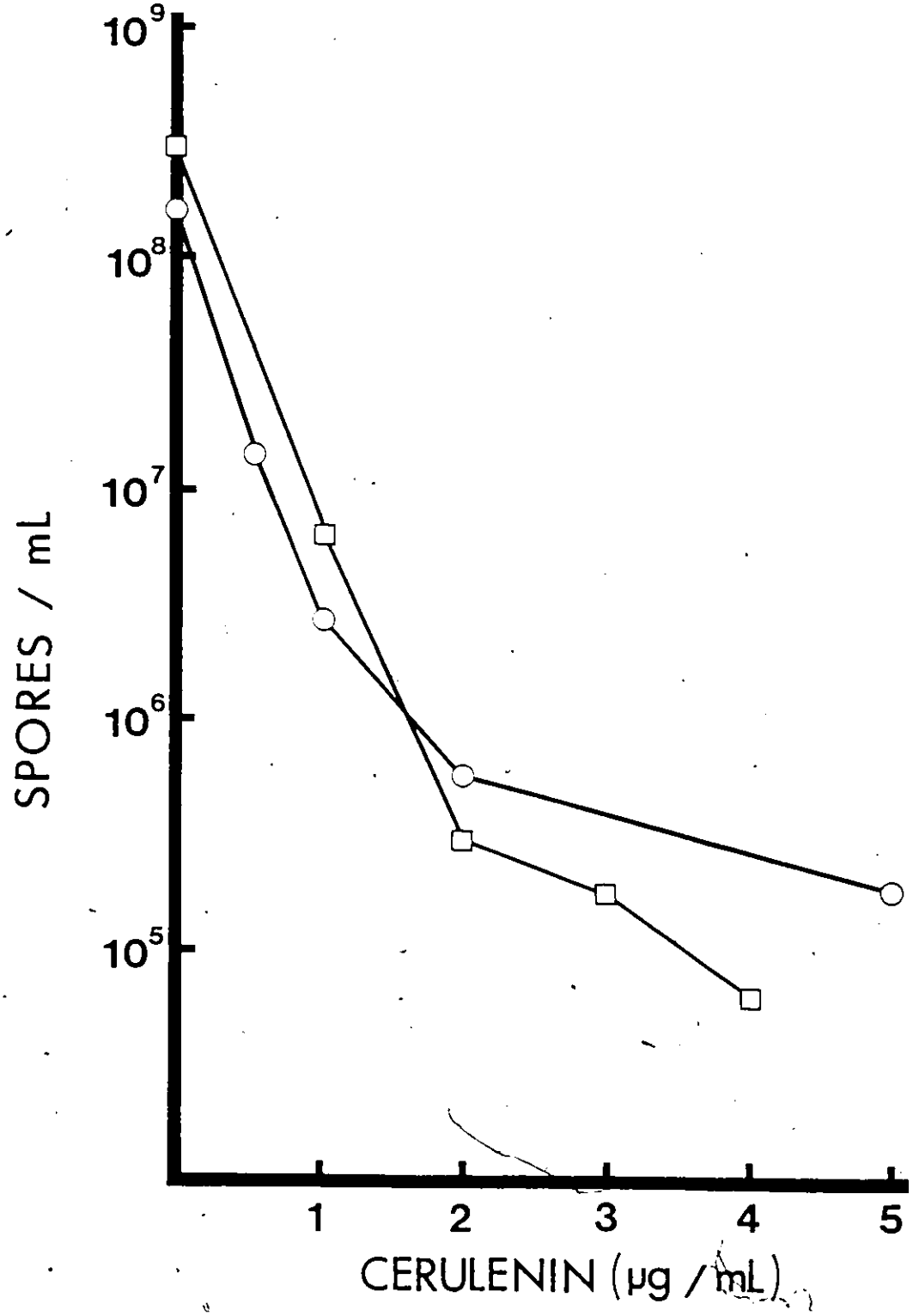

10^9
10^8
10^7
10^6
10^5
SPORES / mL
1
2
3
4
5
CERULENIN (µg / mL)

Table 13. Electron microscopic observation of cells treated with cerulenin

Time of sampling	Stages of sporulation				
	(0+I)	II	III	IV	V
t_2	90	5	5	0	0
t_3	90	2	8	0	0
t_5	88	6	5	1	0
t_7	88	6	5	1	0

Note: Cells of WS (wild-type) were grown in SP medium and cerulenin (1 μg/mL) was added at $t_{-0.5}$. Samples were taken at times indicated in the table and immediately fixed for electron microscopic examination. The results were expressed as the percentage of cells showing different stages of sporulation.

of cells were unable to form spore septum (stage II) in the presence of cerulenin. Since several authors proposed that the filament formation (stage I) may not be required for sporulation (quoted in Robinson and Spotts 1983), the numbers of cells having the morphology of stage 0 and I were presented together. This ultrastructural analysis suggests that cerulenin blocks sporulation at a very early stage.

The stage of sporulation which is sensitive to cerulenin was also determined by adding the drug to a wild-type culture at different times. The results are summarized in Table 14. It appears that sporulation of strain WS is very sensitive to cerulenin at t_0 and it becomes less sensitive after $t_{1.0}$. On the contrary, strain GLU-47 sporulated normally regardless of the time of addition of cerulenin (Table 14).

Table 15 summarizes the sensitivity of various crs mutants to cerulenin. In addition to GLU-47 (crsA47), other crs mutants such as GLN-2 (crsC2), MAN-B1 (crsE1) and MAL-4 (crsF4) were also able to sporulate in the presence of cerulenin. On the other hand, sporulation of mutant strains RIB-2 (crsC1) and MAN-A1 (crsD1) was sensitive to this antibiotic. Cerulenin at 1 µg/mL had practically no effect on the viability of the wild-type strain (WS) as well as those of the crs mutant strains tested.

To investigate the effect of ethanol on sporulation

Table 14. Effect of cerulenin introduced into cultures at different times

Time of addition	WS (wild-type)		GLU-47	
	Viable cell count	Spore count	Viable cell count	Spore count
$t_{-1.0}$	5.3×10^8	4.0×10^5	4.6×10^8	4.4×10^8
$t_{-0.5}$	5.8×10^8	2.6×10^6	3.5×10^8	4.5×10^8
t_0	3.6×10^8	5.0×10^4	4.5×10^8	3.5×10^8
$t_{1.0}$	5.9×10^8	1.9×10^6	3.5×10^8	3.5×10^8
$t_{2.0}$	4.5×10^8	2.1×10^7	5.0×10^8	3.5×10^8
no cerulenin	5.5×10^8	1.1×10^8	5.1×10^8	4.1×10^8

Note: Cells were grown in SP medium and at different times cerulenin was added at 1 µg/mL. The numbers of viable cells and spores were determined after 24 h.

Table 15. Effect of cerulenin on sporulation of crs mutants

Strain	Viable cell count (V)	Spore count (S)	S/V
RIB-2 (crsC1)	4.0×10^{8}	1.6×10^{6}	4.0×10^{-3}
GLN-2 (crsC2)	1.4×10^{8}	9.1×10^{7}	6.5×10^{-1}
MAN-A1 (crsD1)	3.6×10^{8}	1.2×10^{7}	3.3×10^{-2}
MAN-B1 (crsE1)	3.4×10^{8}	1.8×10^{8}	5.3×10^{-1}
MAL-4 (crsF4)	4.2×10^{8}	3.2×10^{8}	7.6×10^{-1}
WS (wils-type)	5.8×10^{8}	2.6×10^{6}	4.5×10^{-3}

Note: Cells were grown in SP medium and at $t_{-0.5}$ cerulenin was added at 1 µg/mL. The numbers of viable cells and spores were determined after 24 h.

of crs mutants, cells were grown in SP medium and ethanol (0.7M) was added to the cultures at $t_{-0.5}$. The numbers of viable cells and spores were determined after 24 h. Mutant strains GLU-47 and MAL-4 were able to sporulate in the presence of ethanol (Table 16). In contrast, sporulation of mutant strains GLN-2, RIB-2 and MAN-A1 and the wild-type strain (WS) was inhibited by this alcohol. Similar results were obtained when methanol (1.1M) or isopropanol (0.5M) was used in place of ethanol (data not shown). On the other hand, vegetative growth of all the strains tested was equally sensitive to these alcohols at concentrations used in the previous experiments.

In our preliminary experiments it was found that the presence of NaCl at 0.85M greatly inhibited sporulation of wild-type strains. This concentration of NaCl had practically no inhibitory effect on viability of the cells. The effect of NaCl on sporulation of crs mutants was then investigated. Cells were grown in SP medium and at $t_{-0.5}$ sterile solid NaCl was added to the cultures. Results presented in Table 17 show that mutant strains GLU-47, MAN-A1, MAN-B1 and MAL-4 are able to sporulate in the presence of NaCl at 0.85M, while strains RIB-2 and GLN-2 are unable to sporulate normally under the same condition.

The effect of NaCl on sporulation of GLU-47 was investigated by adding NaCl to the culture at $t_{-0.5}$ and by

Table 16. Effect of ethanol on sporulation of crs mutants

Strain	Viable cell count (V)	Spore count (S)	S/V
GLU-47 (crsA47)	4.6×10^8	9.4×10^7	2.0×10^{-1}
RIB-2 (crsC1)	3.9×10^8	2.1×10^6	5.4×10^{-3}
GLN-2 (crsC2)	2.4×10^8	7.6×10^6	3.1×10^{-2}
MAN-A1 (crsD1)	2.8×10^8	3.0×10^6	1.1×10^{-2}
MAN-B1 (crsE1)	6.1×10^8	4.1×10^7	6.7×10^{-2}
MAL-4 (crsF4)	3.2×10^8	2.8×10^8	8.8×10^{-1}
WS (wild-type)	4.7×10^8	2.5×10^6	5.3×10^{-3}

Note: Cells were grown in SP medium and ethanol was added (0.7M) at $t_{-0.5}$. The numbers of viable cells and spores were determined after 24 h.

Table 17. Effect of NaCl on sporulation of crs mutants

Strain	Viable cell count (V)	Spore count (S)	S/V
GLU-47 (crsA47)	2.5×10^8	2.2×10^8	8.8×10^{-1}
RIB-2 (crsC1)	1.5×10^8	1.4×10^6	9.3×10^{-3}
GLN-2 (crsC2)	1.3×10^8	3.1×10^6	2.4×10^{-2}
MAN-A1 (crsD1)	9.7×10^7	2.1×10^7	2.2×10^{-1}
MAN-B1 (crsE1)	1.6×10^8	6.1×10^7	3.8×10^{-1}
MAL-4 (crsF4)	2.2×10^8	1.3×10^8	5.9×10^{-1}
WS (wild-type)	1.7×10^8	5.5×10^5	3.2×10^{-3}

Note: Cells were grown in SP medium and solid NaCl (0.85M) was added at $t_{-0.5}$. The numbers of viable cells and spores were determined after 24 h.

estimating the numbers of spores periodically. Although the appearance of mature spores was delayed by the presence of NaCl, the final yield of spores was not affected greatly in the mutant culture (Table 18). Under the same condition a wild-type culture (WS) had a spore count only 5.5×10^5/mL at t_{20}.

To determine the stage of sporulation which is sensitive to NaCl, cells of a wild-type strain (WS) were grown in SP medium and at different times NaCl was added to the culture. The numbers of viable cells and spores were determined after 24 h. The results are summarized in Table 19. During the period between the mid-log phase and $t_{0.5}$ the addition of NaCl reduced greatly the numbers of spores. During the period between $t_{1.0}$ and $t_{4.0}$, both the numbers of viable cells and spores were reduced greatly by NaCl. At present, it is not clear why sporulation is relatively insensitive to NaCl at $t_{1.0}$.

Table 18. Effect of NaCl on sporulation of GLU-47

Time of sampling	GLU-47 +NaCl	GLU-47 -NaCl	WS (wild-type) -NaCl
t_4	4.0×10^4	2.4×10^6	1.5×10^2
t_6	5.2×10^4	1.3×10^8	1.5×10^7
t_8	1.0×10^6	2.1×10^8	7.3×10^7
t_{10}	1.1×10^7	3.8×10^8	-
t_{20}	1.3×10^8	5.1×10^8	1.7×10^8

Note: Cells were grown in SP medium and at $t_{-0.5}$ NaCl was added at 0.85M. The results were expressed as the number of spores per mL.

Table 19. Effect of NaCl added at different times on sporulation

Time of addition	Viable cell count (V)	Spore count (S)	S/V
$t_{-1.5}$	9.8×10^{7}	5.6×10^{4}	5.7×10^{-4}
$t_{-0.5}$	9.6×10^{7}	3.6×10^{5}	3.8×10^{-3}
$t_{0.5}$	1.7×10^{8}	4.7×10^{5}	2.8×10^{-3}
$t_{1.0}$	1.8×10^{8}	2.8×10^{7}	1.6×10^{-1}
$t_{2.0}$	8.0×10^{7}	2.4×10^{4}	3.0×10^{-4}
$t_{4.0}$	8.0×10^{6}	2.0×10^{4}	2.5×10^{-3}
$t_{5.0}$	9.0×10^{6}	1.3×10^{5}	1.4×10^{-2}
No NaCl added	5.9×10^{8}	3.3×10^{8}	5.6×10^{-1}

Note: Cells of a wild-type strain (WS) were grown in SP medium and at the times indicated NaCl was added at 0.85M. The numbers of viable cells and spores were determined after 24 h.

Chapter V

RELATIONS BETWEEN THE crs MUTATIONS AND OTHER MUTATIONS

Suppressor mutations are useful in determining the function affected by a mutation and relations between groups of mutations (Hartman and Roth 1973; Sharrock and Leighton 1981). In the present study, attempts were made to isolate suppressor-carrying mutants from a mutant strain GLU-47 (crsA47). In addition, relations between crs mutations and other mutations which are known to affect various cellular functions were also examined.

1. Suppression of crs mutations by sca mutations

(1). Isolation of suppressor-carrying mutants from GLU-47

To isolate suppressor-carrying mutants, cells of GLU-47 (crsA47) were treated with methylmethanesulfonate as described in Chapter II. From the treated cultures, two Crs^S strains, CS122 and CS123 were isolated. These two mutants sporulated well in SBM containing glucose at 11mM but did so poorly when glucose concentration was raised to 55mM (Table 20).

To test the presence of the original crs mutation in

Table 20. Sporulation of suppressor mutants in SBM

Strain	Glucose (mM)	Viable cell count (V)	Spore count (S)	S/V
GLU-47	55	8.5×10^{8}	6.0×10^{8}	7.1×10^{-1}
(*crsA47*)	11	5.8×10^{8}	4.5×10^{8}	7.8×10^{-1}
CS122	55	1.3×10^{9}	8.5×10^{5}	6.5×10^{-4}
(*crsA47 sca19*)	11	1.0×10^{9}	3.4×10^{8}	3.4×10^{-1}
CS123	55	2.4×10^{9}	1.3×10^{7}	5.4×10^{-3}
(*crsA47 sca27*)	11	1.6×10^{9}	2.7×10^{8}	1.8×10^{-1}

Note: Cells were grown in SBM containing glucose at concentrations indicated. The numbers of viable cells and spores were determined after 24 h.

CS122 and CS123, one of Dedonder's Kit strains that carried aroD120 and lys1 was transduced with lysates of CS122 and CS123. It was found that about 30-40% of Aro^+ transductants were resistant to glucose for sporulation. This indicates that the original crsA47 mutation is still present in strains CS122 and CS123. The two suppressor mutations, sca19 (CS122) and sca27 (CS123) are probably intergenic suppressors since they are not linked to crsA47 by transduction.

Previous results showed that sporulation of GLU-47 was resistant to inhibitory agents such as novobiocin, cerulenin, ethanol and NaCl. To determine whether the suppressor mutations could suppress not only the Crs^R phenotype of GLU-47 but also resistance to these inhibitors, properties of double mutants carrying crsA47 and one of the suppressor mutations were investigated. Cells of CS122 (crsA47 sca19) and CS123 (crsA47 sca27) were grown in SP medium and the inhibitory agents were added to the cultures at $t_{-0.5}$. As shown in Table 21, sporulation of both strains was sensitive to all the agents tested. Therefore, sca19 and sca27 suppress not only resistance to glucose but also resistance to these inhibitors.

(2). Growth, glucose utilization and enzyme activities of sca-carrying mutants

It was found earlier that growth rate and the rate

Table 21. Effect of various inhibitory agents on sporulation of suppressor mutants

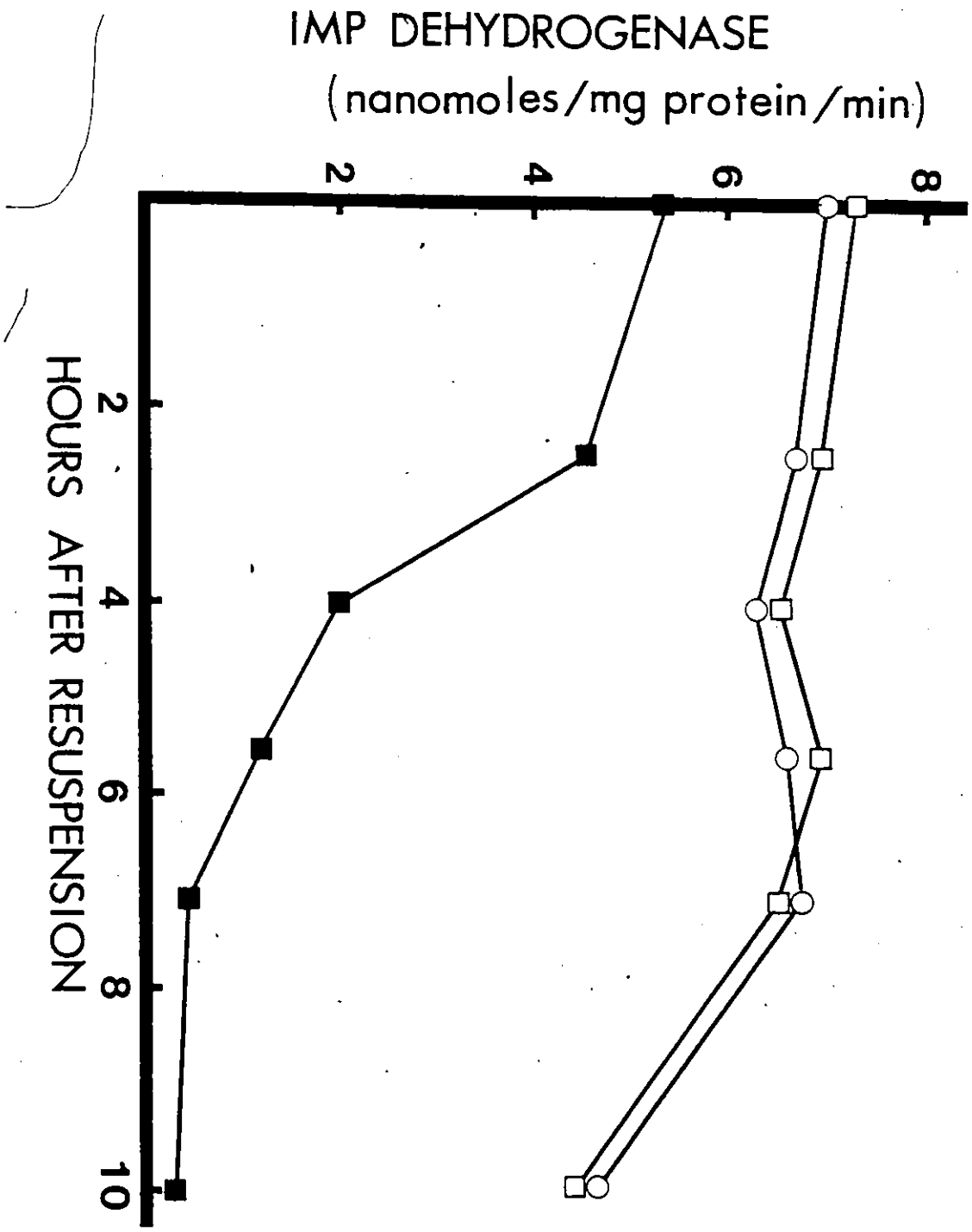
Strain	Inhibitory agent	Viable cell count (V)	Spore count (S)	S/V
CS122 (crsA47 sca19)	None	4.3×10^{8}	2.1×10^{8}	4.9×10^{-1}
	Novobiocin	7.7×10^{7}	5.0×10^{4}	6.5×10^{-4}
	Ethanol	3.7×10^{8}	1.6×10^{5}	4.3×10^{-4}
	Cerulenin	3.3×10^{8}	2.0×10^{7}	6.7×10^{-2}
	NaCl	2.2×10^{8}	8.4×10^{6}	3.8×10^{-2}
CS123 (crsA47 sca27)	None	6.6×10^{8}	9.7×10^{7}	1.5×10^{-1}
	Novobiocin	6.0×10^{7}	5.5×10^{5}	9.2×10^{-3}
	Ethanol	5.1×10^{8}	3.0×10^{5}	5.9×10^{-4}
	Cerulenin	5.6×10^{8}	3.2×10^{6}	5.7×10^{-3}
	NaCl	1.1×10^{8}	7.5×10^{5}	6.3×10^{-3}

Note: Cells were grown in SP medium and novobiocin (5 µg/mL), ethanol (0.7M), cerulenin (1 µg/mL) or NaCl (0.85M) was added at $t_{-0.5}$. The numbers of viable cells and spores were determined after 24 h.

of glucose utilization of crsA47 mutant were much lower than those of a wild-type strain (WS). Experiments were carried out to determine whether the suppressor mutations could alter the rate of growth and glucose utilization in crsA47-carrying strains. It was found that doubling times of CS122 and CS123 were 72 min and 75 min, respectively in SBM containing glucose. These values indicate that the suppressor mutants grow at a much faster rate than the parental strain GLU-47 does. The rate of glucose utilization was determined in SBM containing glucose by the resuspension technique. After 16 h of incubation, glucose was almost completely used up in both CS122 and CS123, whereas only about 40% of the original amount of glucose was metabolized by GLU-47 during the same period.

Earlier experiments revealed that the activity of IMP dehydrogenase in GLU-47 was lower than that in the wild-type strain (WS). The presence of glucose greatly inhibited the decrease in the enzyme activity in the wild-type strain but not in GLU-47. To determine the effect of sca mutations on this enzyme, the enzyme activities in double mutants that carried crsA47 and an sca mutation were determined. Cells were grown in SP medium. At $t_{-0.5}$ specific activities of IMP dehydrogenase in CS122 (crsA47 sca19) and CS123 (crsA47 sca27) were considerably higher than that in GLU-47 (crsA47) (Fig. 9). After resuspension of cells in SBM containing

Fig. 9. Specific activity of IMP dehydrogenase in strains CS122, CS123 and GLU-47. Cells were grown in SP medium. At $t_{-0.5}$ the cells were resuspended in SBM containing glucose (55mM). ○, CS122; □, CS123; ■, GLU-47.


IMP DEHYDROGENASE
(nanomoles/mg protein/min)
2
4
6
8
HOURS AFTER RESUSPENSION
2
4
6
8
10

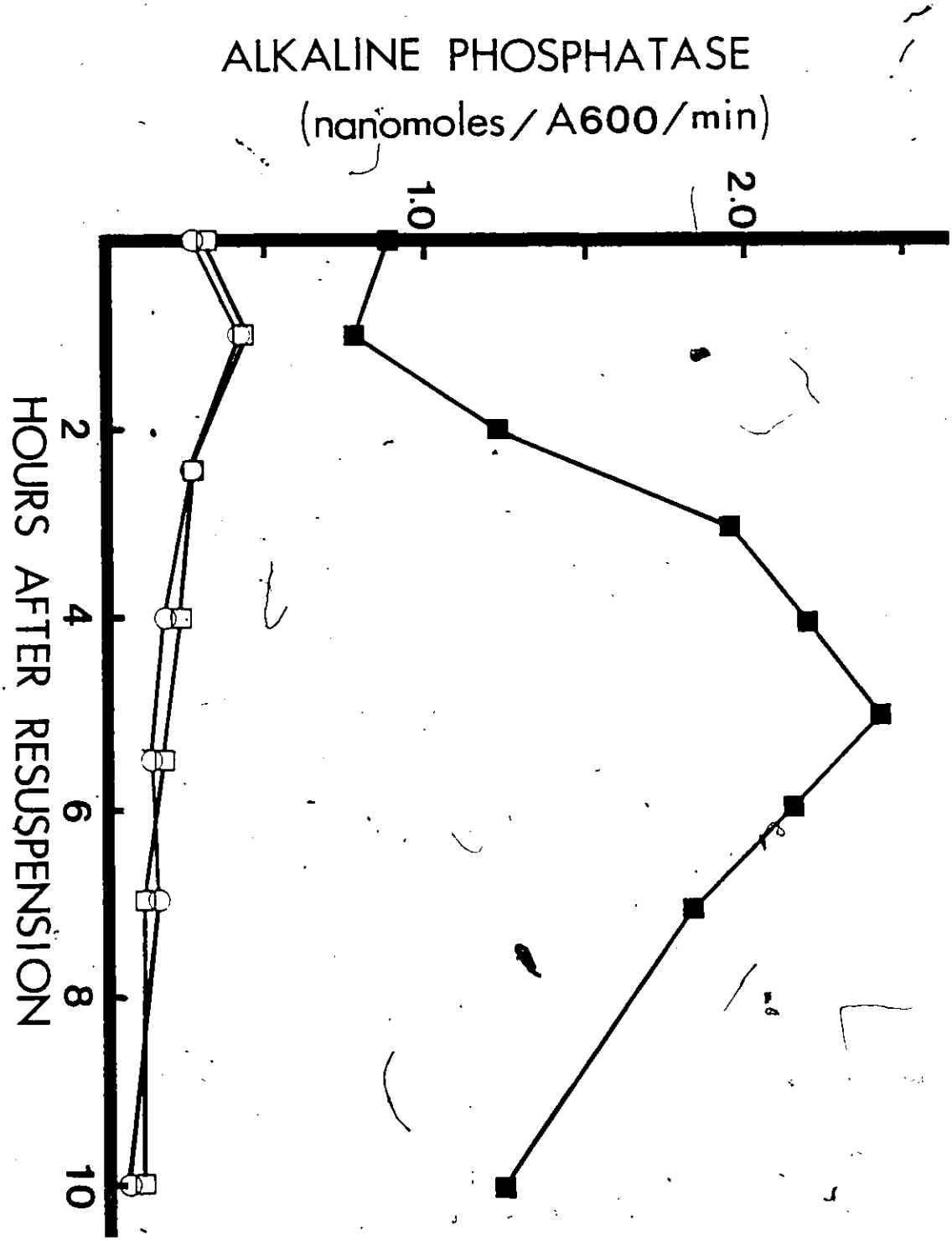
glucose, the enzyme levels in CS122 and CS123 failed to show the significant decrease that occured in GLU-47 (Fig. 9).

As for alkaline phosphatase, it was found that the activity of this enzyme in GLU-47 increased rapidly after resuspension of cells in SBM regardless of the presence or absence of glucose. In order to determine the levels of this enzyme in CS122 and CS123, cells were grown in SP medium supplemented with phosphate buffer (10mM), pH7.0. Cells were then resuspended and incubated in SBM containing glucose. At the time of resuspension, background levels of alkaline phosphatase in both CS122 and CS123 were only about one third of that in GLU-47 (Fig. 10). In contrast to the rapid increase in specific activity of the enzyme in GLU-47, the enzyme activities in strains CS122 and CS123 remained at a very low level throughout the experiment (Fig. 10).

(3) Mapping of *sca* mutations

In order to map these two *sca* mutations, a series of strains that carried both *crsA47* and auxotrophic markers were constructed by introducing *crsA47* mutation into Dedondor's Kit strains (Dedondor *et al*. 1977). The Kit strains were transformed to Str^R with relatively high concentrations (1-2 μg/mL) of DNA extracted from GLU-47 (*strA1 crsA47*). Among the Str^R transformants, those which had received the *crsA47* mutation could be recognized by their

Fig. 10. Specific activity of alkaline phosphatase in strains CS122, CS123 and GLU-47. Cells were grown in SP medium supplemented with phosphate buffer (10mM), pH7.0. At $t_{-0.5}$ the cells were resuspended in SBM containing glucose (55mM). ○, CS122; □, CS123; ■, GLU-47.


ALKALINE PHOSPHATASE
(nanomoles / A600 / min)
1.0
2.0
2
4
6
8
10
HOURS AFTER RESUSPENSION

rough colonial morphology on TBB agar and by their Crs^R phenotype. Colonies having Str^R Crs^R phenotype were then examined for the presence of the auxotrophic markers that were originally carried by their parental Kit strain. The crsA47-carrying Kit strains were then used as recipient and CS122 (crsA47 sca19) or CS123 (crsA47 sca27) as donor in transduction crosses. Prototrophic transductants for the auxotrophic markers carried by the recipient strains were examined for their ability to sporulate in the presence of glucose. It was found that about 10-20% of the prototrophic transductants for aroG932 (CS145) or thr5 (CS146) had the Crs^S phenotype. On the other hand, prototrophic transductants for other auxotrophic markers were still Crs^R. These results indicate that both sca19 and sca27 are linked to aroG932 and thr5 (Table 22). Three-factor transduction crosses were carried out in which CS146 (crsA47 hisA1 thr5) was treated with lysates of CS122 (sca19 crsA47) or CS123 (sca27 crsA47). Thr^+ transductants were selected. If sca mutations were located between hisA1 and thr5, transductants showing Thr^+ His^+ Crs^R phenotype would be produced at the lowest frequency since the formation of these transductants required a double crossover. In fact, the frequencies of Thr^+ His^+ Crs^R transductants were 10-12% whereas no Thr^+ His^+ Crs^S transductants were produced (Table 22). Thus, the gene order may be "hisA1-thr5-sca27-sca19-aroG932" (Fig.11). Since the map positions of aroG932 and thr5 are 270 and 290, respectively (Henner and Hoch 1980), those of sca19 and sca27 were esti-

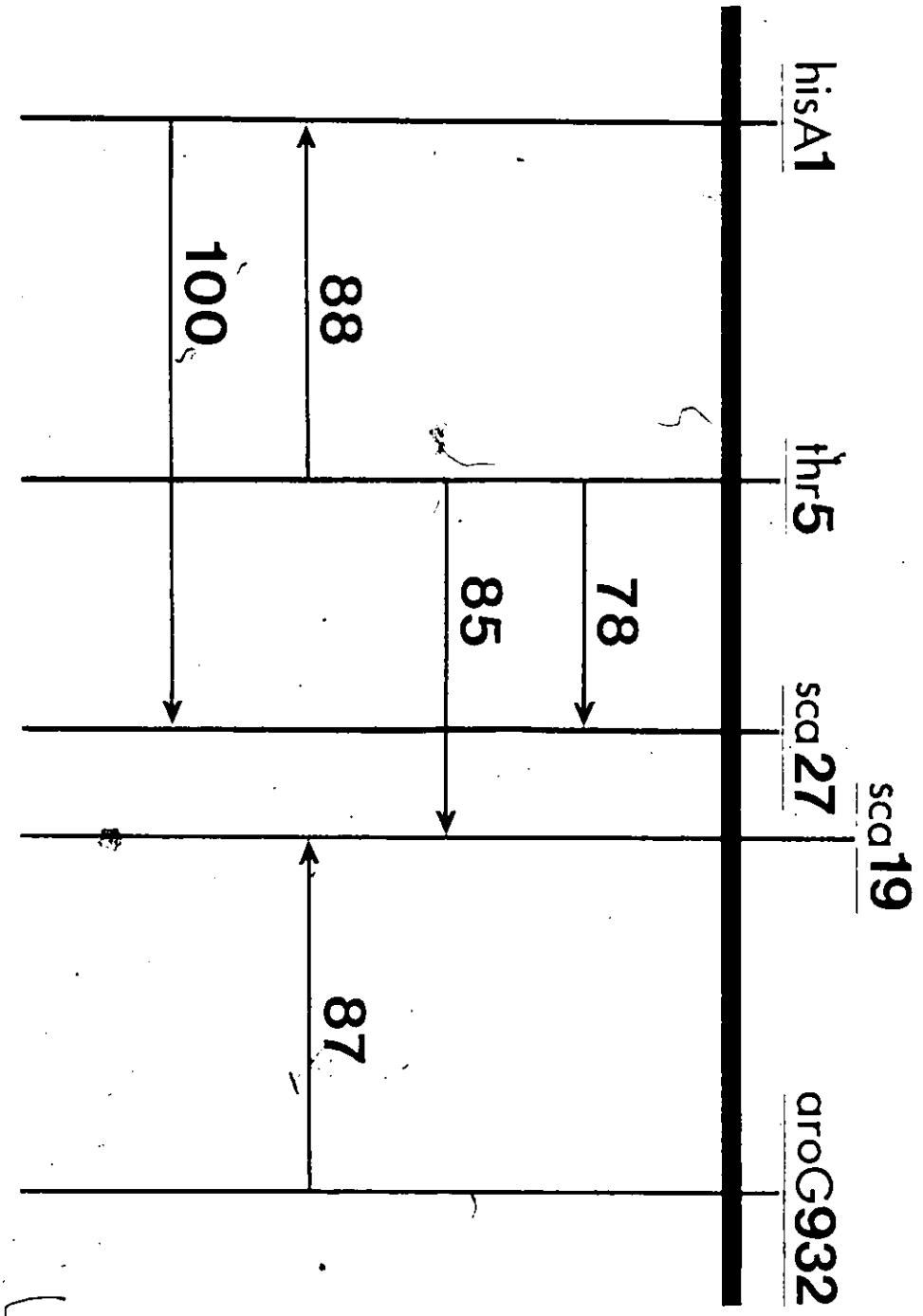
Table 22. Transduction crosses involving sca19, sca27 and neighbouring markers

Recipient genotype	Donor genotye	Selected marker	Recombinants[a] Classes			No. of colonies	Suggested order
aroG932 crsA47 (CS145)	crsA47 sca19 (CS122)	Aro+	Aro 1 1	Sca[b] 0 1		391 58	
			His	Thr	Sca		
hisA1 thr5 crsA47 (CS146)	crsA47 sca19 (CS122)	Thr+	0 0 1 1	1 1 1 1	0 1 0 1	158 33 30 0	hisA1-thr5-sca19
			His	Thr	Sca		
hisA1 thr5 crsA47 (CS146)	crsA47 sca27 (CS123)	Thr+	0 0 1 1	1 1 1 1	0 1 0 1	114 35 17 0	hisA1-thr5-sca27

[a] 0, recipient phenotype; 1, donor phenotype.

[b] Sca, transductants which were sensitive to glucose for sporulation.

Fig. 11. Genetic map showing the positions of scal9 and sca27. The positions were determined by transduction. Mapping unit distance = 100% - percentage of co-transduction. Arrows point from the selected marker to the unselected marker.


hisA1
thr5
sca27
sca19
aroG932
100
88
85
78
87

mated to be approximately 280.

To determine whether the sca mutations were located in the same gene, recombination tests were carried out. Strains WS (wild-type), CS122 (crsA47 sca19) and CS123 (crsA47 sca27) were used as donor. Strain CS318 (aroD120 crsA47 sca27) was transformed with the donor DNA and Aro^+ transformants were selected. It was found that, when CS123 was used as donor, none of 2244 Aro^+ transformants showed Crs^R phenotype. When CS122 was used as donor, however, 15 of 1758 Aro^+ transformants (0.9%) were Crs^R. When WS was used as donor, 31 of 3380 Aro^+ transformants (0.9%) were Crs^R. If sca19 and sca27 were located in the same gene, the Crs^R transformants would be produced at an extremely low frequency in crosses between sca19 and sca27. In fact, the frequency of Crs^R transformants with CS122 as donor was similar to that with WS as donor. It is therefore concluded that these two mutations are not located in the same gene.

(4). Suppression of other crs mutations by sca19

To investigate the effect of sca19 on Crs^R phenotype of crs mutants other than crsA47, double mutants that carried both sca19 and one of the crs mutations were constructed. All the constructed double mutants sporulated poorly in SBM containing glucose (Table 23). This indicates that sca19 can suppress not only Crs^R phenotype of crsA47 but also that of other crs mutations (crsA1, crsA4, crsC1, crsD1, crsE1 and crsF4).

Table 23. Sporulation of strains carrying scal9 and crs mutations

Strain	Viable cell count (V)	Spore count (S)	S/V
WS (wild-type)	3.4×10^{9}	1.1×10^{7}	3.3×10^{-3}
CS249 (scal9 crsA1)	1.5×10^{9}	1.1×10^{6}	7.3×10^{-4}
GLU-1 (crsA1)	1.0×10^{9}	5.3×10^{8}	5.3×10^{-1}
CS250 (scal9 crsA4)	1.8×10^{9}	6.7×10^{5}	3.7×10^{-4}
FRU-4 (crsA4)	6.5×10^{8}	5.0×10^{8}	7.7×10^{-1}
CS306 (scal9 crsC1)	2.4×10^{9}	2.5×10^{3}	1.0×10^{-6}
RIB-2 (crsC1)	2.2×10^{9}	7.5×10^{8}	3.4×10^{-1}
CS341 (scal9 crsD1)	3.2×10^{9}	2.9×10^{5}	9.0×10^{-5}
MAN-A1 (crsD1)	1.8×10^{9}	8.0×10^{8}	4.4×10^{-1}
CS309 (scal9 crsE1)	3.5×10^{9}	4.1×10^{5}	1.2×10^{-4}
MAN-B1 (crsE1)	2.3×10^{9}	5.3×10^{8}	2.3×10^{-1}
CS304 (scal9 crsF4)	1.3×10^{9}	1.2×10^{2}	9.2×10^{-8}
MAL-4 (crsF4)	2.2×10^{9}	6.4×10^{8}	2.9×10^{-1}

Note: Cells were grown in SBM containing glucose at 55mM. The numbers of viable cells and spores were determined after 24 h.

2. Suppression of crs mutations by an rfm mutation

During the previous mapping study of crsE1 mutation (Chapter III) it was found that a double mutant (CS31) which carried crsE1 and rfm11 (rifamycin resistance mutation) was unable to sporulate in the presence of glucose, while another double mutant (rfm1 crsE1) remained glucose resistant. To determine whether suppression of catabolite resistance in strain CS31 was due to the presence of rfm11 mutation, this mutation was introduced into strain MAN-B1 (crsE1) by transformation with DNA extracted from CS31. About 100 Rfm^R transformants tested were catabolite sensitive for sporulation, indicating that rfm11 was indeed acting as a suppressor for crsE1 mutation.

The rfm11 mutation was subsequently introduced into strain FRU-4 (crsA4), RIB-2 (crsC1), GLN-2 (crsC2), MAN-A1 (crsD1) and MAL-4 (crsF4) by transduction with strain CS35 (rfm11 strA1) as donor rather than strain CS31 (rfm11 crsE1) since rfm11 was closely linked to crsE1. Transductants showing the Rfm^R Str^R phenotype were selected to ensure that the Rfm^R phenotype observed was due to the rfm11 mutation and not due to a spontaneous rfm mutation. By this technique, strains CS36 (rfm11 strA1 crsA4), CS37 (rfm11 strA1 crsC1), CS41 (rfm11 strA1 crsC2), CS38 (rfm11 strA1 crsF4), and CS39 (rfm11 strA1 crsD1) were constructed. An-

other strain, CS40 (rfm11 crsA47) was obtained by transducing GLU-47 (strA1 crsA47) with lysates of CS33 (rfm11) and by selecting Rfm^R Str^S recombinants. It was found that the constructed rfm11 mutants carrying crsA4 or crsA47 mutations sporulated normally in SBM containing glucose, while those carrying crsC1, crsC2, crsD1 or crsF4 mutations did so poorly under the same condition (Table 24). It can be concluded that rfm11 can suppress the Crs^R phenotype of crsC1, crsC2, crsD1, crsE1 and crsF4 but not that of crsA4 and crsA47.

3. Relation between eryl and crs mutations

Sharrock and Leighton (1981, 1982) reported that an intergenic suppressor mutation, rev4 could confer resistance to the inhibitory effect of cerulenin and ethanol on sporulation. It would be of interest to know whether this mutation can also confer resistance to catabolites for sporulation. Sharrock and Leighton's original strain CB2401 which carried both rev4 and eryl mutations was first tested for the ability to sporulate in SBM containing glucose. It was found that strain CB2401 (rev4 eryl) sporulated poorly in the presence of glucose and that a single mutant CB400 which carried eryl only also sporulated poorly under the same condition. As eryl is known to cause Spo^{ts} phenotype (Sharrock and Leighton 1981), the failure of strain CB2401

Table 24. Sporulation of strains carrying *rfm11* and *crs* mutations

Strain	Viable cell count (V)	Spore count (S)	S/V
WS (wild-type)	3.4×10^{9}	1.1×10^{7}	3.3×10^{-3}
CS33 (*rfm11*)	1.4×10^{9}	3.7×10^{4}	2.6×10^{-5}
CS40 (*rfm11 crsA47*)	9.0×10^{8}	2.1×10^{8}	2.3×10^{-1}
CS36 (*rfm11 crsA4*)	9.4×10^{8}	4.2×10^{8}	4.5×10^{-1}
CS37 (*rfm11 crsC1*)	4.8×10^{9}	1.3×10^{6}	2.7×10^{-4}
CS41 (*rfm11 crsC2*)	2.2×10^{9}	1.5×10^{6}	6.8×10^{-4}
CS39 (*rfm11 crsD1*)	1.3×10^{9}	4.9×10^{6}	3.8×10^{-3}
CS31 (*rfm11 crsE1*)	2.8×10^{8}	1.1×10^{6}	3.9×10^{-3}
CS38 (*rfm11 crsF4*)	2.4×10^{9}	1.5×10^{6}	6.3×10^{-4}

Note: Cells were grown in SBM containing glucose at 55mM. The numbers of viable cells and spores were determined after 24 h.

to show Crs^R phenotype might be due to the presence of eryl mutation. Attempts were then made to construct a strain that carried rev4 alone. Strain CB2401 (eryl rev4) was treated with lysates of WS (strA1) and Str^R Ery^S transductants were selected. The constructed strain, CS327 (strA1 rev4) sporulated well in SBM containing glucose (Table 25). It can be concluded from these results that rev4 can confer Crs^R phenotype and that eryl is able to suppress the Crs^R phenotype of rev4 mutant.

The effect of eryl mutation on Crs^R phenotype of our crs mutants was then determined. Double mutant strains, CS330 (eryl crsA47), CS331 (eryl crsC1), CS332 (eryl crsD1), CS337 (eryl crsF4) and CS338 (eryl crsE1) were constructed by transduction or by transformation. It was found that Crs^R phenotype of double mutants carrying crsC1, crsD1, crsE1 or crsF4 was suppressed by eryl mutation (Table 26). The only crs mutation which was not affected by eryl was crsA47 (Table 26).

Sharrock and Leighton (1981) showed that rev4 could suppress Spo^{ts} phenotype caused by eryl mutation. In order to determine whether our crs mutations have a similar suppressing effect, the above eryl crs double mutants were grown in 2xSG medium at 47°C and the number of viable cells and spores were determined at t_{10}. Results shown in Table 27 indicate that the Spo^{ts} phenotype caused by eryl can be sup-

Table 25. Effect of glucose on sporulation of strains carrying rev4 mutation

Strain	Viable cell count (V)	Spore count (S)	S/V
CB400 (eryl)	2.3×10^{9}	8.9×10^{6}	3.8×10^{-3}
CB2401 (eryl rev4)	3.1×10^{9}	4.0×10^{7}	1.3×10^{-2}
CS327 (strA1 rev4)	1.7×10^{9}	8.5×10^{8}	5.0×10^{-1}

Note: Cells were grown in SBM containing glucose at 55mM. The numbers of viable cells and spores were determined after 24 h.

Table 26. Effect of glucose on sporulation of various *eryl crs* strains

Strain	Viable cell count (V)	Spore count (S)	S/V
CS330 (*eryl crsA47*)	6.6×10^{8}	8.6×10^{7}	1.3×10^{-1}
CS331 (*eryl crsC1*)	2.9×10^{9}	1.7×10^{7}	5.9×10^{-3}
CS332 (*eryl crsD1*)	2.3×10^{9}	1.6×10^{5}	7.0×10^{-5}
CS338 (*eryl crsE1*)	2.5×10^{9}	3.8×10^{7}	1.5×10^{-2}
CS337 (*eryl crsF4*)	4.0×10^{9}	5.0×10^{1}	1.3×10^{-8}

Note: Cells were grown in SBM containing glucose at 55mM. The numbers of viable cells and spores were determined after 24 h.

Tbale 27. Effect of high temperature (47°C) on sporulation of eryl crs strains

Strain	Viable cell count (V)	Spore count (S)	S/V
CB400 (eryl)	5.1×10^{8}	2.3×10^{7}	5.9×10^{-2}
CS330 (eryl crsA47)	1.2×10^{8}	1.0×10^{8}	8.3×10^{-1}
CS331 (eryl crsC1)	2.5×10^{8}	4.5×10^{6}	1.8×10^{-2}
CS332 (eryl crsD1)	3.2×10^{8}	7.8×10^{5}	2.4×10^{-3}
CS338 (eryl crsE1)	2.9×10^{8}	1.9×10^{8}	6.6×10^{-1}
CS337 (eryl crsF4)	1.0×10^{8}	2.5×10^{7}	2.5×10^{-1}

Note: Cells were grown in 2xSG medium at 47°C. The numbers of viable cells and spores were determined at t_{10}.

pressed by crsA47, crsE1 and crsF4 but not by crsC1 and crsD1.

4. Stringent response and Crs^R phenotype

It was reported that stringent response was not absolutely required for sporulation since relaxed mutants (relA) sporulated normally (Rhaese et al. 1975; Nishino et al. 1979). However, sporulation could be initiated by the stringent response to partial amino acid deprivation (Lopez et al. 1981b). Endo et al. (1983) described a mutant strain in which partial stringent response caused cells to sporulate in the presence of glucose. In the present study, the relation between the stringent response and Crs^R phenotype of our crs mutants was investigated. The relA mutation carried by strain IS56 (Smith et al. 1980) was introduced into our crs strains by transduction and transformation. Recombinants that carried the relA mutation were identified by the procedure described in Chapter II. The relA crs double mutants were then examined for their ability to sporulate in SBM containing glucose. It was found that double mutants carrying relA and one of crsA47, crsC1, crsE1 and crsF4 were able to sporulate in SBM containing glucose (Table 28). Strain CS365 (relA crsD1) was sensitive to glucose (Table 28). These results suggest that the stringent response is

Table 28. Effect of glucose on sporulation of relA crs strains

Strain	Viable cell count (V)	Spore count (S)	S/V
CS384 (relA trpC2)	3.0×10^9	2.2×10^7	7.3×10^{-3}
CS410 (relA crsA47 trpC2)	1.0×10^9	5.1×10^8	5.1×10^{-1}
CS387 (relA crsC1 trpC2)	2.5×10^9	8.8×10^8	3.5×10^{-1}
CS365 (relA crsD1 trpC2)	2.2×10^9	2.0×10^7	9.1×10^{-3}
CS391 (relA crsE1 trpC2)	3.2×10^9	1.0×10^9	3.1×10^{-1}
CS395 (relA crsF4 trpC2)	2.6×10^9	1.3×10^9	5.0×10^{-1}

Note: Cells were grown in SBM containing glucose at 55mM. The numbers of viable cells and spores were determined after 24 h.

necessary for the Crs^R phenotype of crsD1 mutant but not for that of crsA47, crsC1, crsE1 and crsF4 mutants.

5. Crs^R phenotype of stv and std mutants

Earlier mapping studies (Chapter III) indicated that the crsE1 mutation was located between std and stv mutations. Since std and stv fall within the rpoBC operon which regulates the synthesis of β and β' subunits of RNA polymerase (Halling et al. 1977, 1978; Doi 1982), crsE1 may also be located in this operon. If this is the case, it would be possible that certain mutations in this operon might show Crs^R phenotype. Attempts were made to isolate mutant strains that carried rfm, stv, and std mutations to investigate the relationship between catabolite resistance and these mutations. A wild-type strain (WS) was treated with EMS and plated on TBB agar containing streptovaricin or streptolydigin to isolate resistant mutants. Stv^R and Std^R colonies thus obtained were replica-plated onto SBM agar containing glucose. After 3 days of incubation, colonies that showed dark-brown pigmentation were selected for further study. In liquid SBM containing glucose, the frequencies of spores of these Stv^R and Std^R mutants ranged from 7.6×10^{-2} to 9.7×10^{-2}, while that of WS was only 8.9×10^{-3} (Table 29). Transformation analysis in which WS was used as recipient and these

Table 29. Effect of stv and std mutations on sporulation

Strain	Viable cell count (V)	Spore count (S)	S/V
WS (wild-type)	2.7×10^9	2.4×10^7	8.9×10^{-3}
CS225 (stv53)	2.9×10^9	2.8×10^8	9.7×10^{-2}
CS256 (std201)	3.3×10^9	2.5×10^8	7.6×10^{-2}
CS277 (stv53 std201)	2.8×10^9	4.2×10^8	1.5×10^{-1}
CS294 (stv339 std201)	2.5×10^9	6.6×10^8	2.6×10^{-1}

Note: cells were grown in SBM containing glucose at 55mM. The numbers of viable cells and spores were determined after 24 h.

mutants as donor showed that such "partial resistance" to glucose and the Stv^R or Std^R phenotype could not be separated from each other. However, none of the Rfm^R mutants isolated from the EMS-treated WS cells showed such partial resistance to glucose.

The catabolite resistance of double mutants carrying both stv and std mutations was then investigated. Std^R Stv^R double mutants were constructed by transformation in which strain CS256 (std201) was used as recipient and CS225 (stv53) as donor. The frequency of spores of a double mutant CS277 (std201 stv53) in SBM containing glucose was 1.5×10^{-1}, which was slightly higher than those of the parental strains (Table 29).

Double mutants having Std^R Stv^R phenotype were also obtained by treating strain CS256 (std201) with EMS. One of the double mutants, CS294 (std201 stv339), sporulated at a frequency of 2.6×10^{-1} in the presence of glucose (Table 29). Mutants showing Crs^R phenotype among the double mutants obtained occured at a frequency of about 1%. On the other hand, the same treatment of strain CS225 (stv53) failed to produce double mutants showing the Crs^R phenotype. These observations indicate that only certain stv or std mutants or certain stv std double mutants are able to sporulate in the presence of glucose.

Chapter VI

DISCUSSION

1. Genetic mapping of crs mutants

Mutants of B. subtilis that are able to sporulate in the presence of inhibitory concentrations of catabolites have been isolated by many investigators (Freese et al. 1970; Ito and Spizizen 1973; Kunst et al. 1974; Ohné and Rutberg 1976; Dod and Balassa 1978; Takahashi 1979). In the present study, a number of crs mutations carried by the catabolite resistant mutants isolated by Takahashi (1979) have been mapped on the chromosome of B. subtilis.

Ito and Spizizen (1973) have isolated several catA mutants which are insensitive to glucose in sporulation. These mutants produce extracellular protease at a level five to six times higher than that of wild-type strains. Dod and Balassa (1978) have suggested that their scoC mutants may be identical with the catA mutants. The mutant gene catA has been found to be linked to metD by PBS1 transduction (Ito and Spizizen 1973). Since the map position of metD has been reported to be 95, the map position of catA (scoC) would be approximately 90 (Henner and Hoch 1980).

The $sacU^h$ mutants isolated by Kunst et al. (1974)

are capable of sporulating in the presence of glucose and they are hyperproducers of extracellular protease. The *sacU*^{h} gene is located at a map position of 310. From the map positions of different catabolite resistance genes summarized in Table 30, it is clear that our *crs* mutations are genetically distinct from the *catA* (*scoC*) and the *sacU*^{h} mutations described by the above authors. Furthermore, unlike the *catA* (*scoC*) and the *sacU*^{h} mutants, our *crs* mutants are not hyperproducers of extracellular protease (I. Takahashi unpublished data).

According to results of our mapping study, *crs* mutations are not clustered at one region of chromosome; they are distributed randomly at six distinct loci (Table 30). Although all the *crs* mutants examined in the present study are able to sporulate in the presence of glucose, they show varying degree of sensitivity to glucose in induction of an inducible enzyme, acetoin dehydrogenase (Takahashi 1979). They also show different sensitivity to novobiocin, acridine orange (Takahashi and MacKenzie 1982), cerulenin, aliphatic alcohols and NaCl in sporulation. From these results, it is suggested that, although the *crs* mutants show the same Crs^{R} phenotype, the functions that are affected in these mutants may be different from each other.

Several authors have reported the existence of other types of catabolite resistant mutants. It was found in the

Table 30. Map positions of various catabolite resistance genes

Mutant genes mapped in the present study		Mutant genes mapped by other authors	
Genotype (strain)	map position	genotype	map position[a]
crsD1 (MAN-A1)	5	*catA*	90
crsE1 (MAN-B1)	10	*scoC*	90
crsB40 (GLU-40)	55	*sacU*^{h}	310
crsF4 (MAL-4)	120		
crsC1 (RIB-2)	220		
crsC2 (GLN-2)	220		
crsA1 (GLU-1)	225		
crsA4 (FRU-4)	225		
crsA47 (GLU-47)	225		

[a] The map positions were estimated from the positions of genetic markers to which *catA*, *scoC* and *sacU*^{h} were linked by PBS1 transduction (Henner and Hoch 1980). *catA*: Ito and Spizizen (1973); *scoC*: Dod and Balassa (1978); *sacU*^{h}: Kunst *et al*. (1974).

present study that the rev4 mutant whose mutation was identified as a suppressor for Spo^{ts} phenotype of eryl-carrying mutants was able to sporulate in the presence of glucose. The rev4 mutation is located between narA (map position: 320) and sacA (map position: 335) (Sharrock and Leighton 1981). Some leaky guanine-requiring mutants have been shown to sporulate well in the presence of excess glucose when guanine supply is limited (Freese et al. 1978, 1979b). Freese et al. (1970) have reported that a mutant which lacks phosphoenolpyruvate transferase activity sporulates normally in the presence of glucose. Ohné and Rutberg (1976) have isolated a mutant which is resistant to glucose and malate for sporulation. These mutants are found to be unable to metabolize either fructose (Freese et al. 1970) or malate (Ohné and Rutberg 1976) as the sole carbon source. As crs mutants can use both fructose and malate as the sole carbon source (Takahashi 1979), the crs mutants appear to be distinct from these mutants.

One of the crs mutations, crsE1, was mapped between rfm and std mutations. Halling et al. (1977) have reported that rfm mutations affect the rpoB gene which codes for the β subunit of RNA polymerase. The std mutations, on the other hand, affect the rpoC gene which codes for the β' subunit of the enzyme (Halling et al. 1978). The rpoB and rpoC gene have been suggested to form an operon (rpoBC) (Doi

1982). Although the RNA polymerase subunits of strains carrying the rfm, stv, std and crsE1 mutations used in the present study have not been examined, it is very likely, from their map positions, that these mutations also fall within the rpoBC operon.

Mutations in the rpoBC operon often produce pleiotropic effects in B. subtilis. Sonenshein et al. (1974) have reported that some rfm and std mutations lead to Spo$^-$ or Spo^{ts} phenotypes. Ryu and Takayanagi (1979) have reported fifteen rfm mutants which are deficient in glutamate synthase. Sporulation in some of the mutant strains is blocked at stage 0, but not in other strains. These authors have concluded that a certain modification of RNA polymerase may affect the expression of the glutamate synthase gene. Hirochika and Kobayashi (1978) have found that an rfm mutation can suppress Spo^{ts} phenotype of a mutant affected in the elongation factor G for protein synthesis. As the crsE1 mutation was located in the rpoBC operon, it was expected that this mutation could confer resistance to RNA polymerase-affecting antibiotics. In fact, vegetative growth of strain MAN-B1 (crsE1) was sensitive to rifamycin, streptovaricin and streptolydigin. However, this observation does not rule out the possibility that crsE1 might confer resistance to a hitherto unidentified antibiotic. It is possible that the resistance to catabolites and other inhibitors may be due to

the pleiotropic effect of this mutation.

It has been reported that lpm mutations which confer resistance to lipiarmycin are also located between rfm (stv) and std (Sonenshein et al. 1977). Lipiarmycin is known to block initiation of RNA synthesis by inhibiting the σ-factor-containing RNA polymerase (Sonenshein and Alexander 1979). Some of the lpm mutations can counteract Spo^{ts} phenotype of certain mutants, and consequently the mutant cells regain the ability to sporulate at high temperatures (D.M. Rothstein, Ph.D. these, Tufts University, Boston, Mass., 1977; quoted in Sonenshein et al. 1977). This situation seems to be analogous to the finding that crsE1 mutation could suppress Spo^{ts} phenotype induced by catabolites and other inhibitors. Genetic analysis involving lpm and crsE1 mutations and an investigation on the sensitivity of crsE1-carrying strains to lipiarmycin may yield useful information.

According to R.H. Doi, crsA47 mutation is located in the gene for the σ-factor of RNA polymerase (personal communication). Furthermore, R.H. Doi and co-workers have found a change in the nucleotide sequence in the promoter region of the gene for the σ-factor in crsA47 mutant. The foregoing observation made with crsE1 and crsA47 mutations indicate that certain changes in RNA polymerase may alter the response of cells to the inhibitory effect of catabolites and other in-

hibitors.

2. Properties of crs mutants

In the present study, it was found that mutant strains that carried crsA47 or crsF4 mutation grew at a slower rate than that of the wild-type strain. On the other hand, strains carrying crsC1 or crsD1 mutation grew almost as fast as the wild-type strain did. This observation suggests that slow growth is not a necessary condition for a mutant to achieve Crs^R phenotype.

The slow growth rate and slow rate of glucose utilization of strain GLU-47 (crsA47) appeared to indicate a deficiency in glucose transport or metabolism. However, the growth rates of this mutant with other carbon sources such as glycerol, gluconate, ribose and malate were also lower than those of the wild-type strain. Since cells transport and metabolize these carbon sources through different mechanisms, an alteration in functions other than transport or metabolism of glucose may be responsible for the slow growth of GLU-47.

Freese et al. (1979b) have shown that the presence of mycophenolic acid, an inhibitor of IMP dehydrogenase makes wild-type B. subtilis cells to sporulate in the presence of glucose. Sporulation of leaky guanine auxotrophic mutants

is resistant to glucose when exogenous supply of guanine is limited (Freese et al. 1978, 1979a). These authors have suggested that the ability to sporulate in the presence of glucose, ammonia and phosphate is due to low levels of GDP and GTP. In the present study, it was found that GLU-47 had a much lower activity of IMP dehydrogenase at the time of cell resuspension as well as during incubation in SBM containing glucose. Considering the fact that IMP dehydrogenase is the first enzyme for the synthesis of GMP from IMP, the above observation seems to suggest that the slow growth rate and Crs^R phenotype of GLU-47 are the results of the low activity of this enzyme. However, the addition of guanosine, which is readily transported into B. subtilis cells (quoted in Freese et al. 1979b), could not increase the growth rate of strain GLU-47, neither could it suppress Crs^R phenotype (data not shown). In addition, specific activities of IMP dehydrogenase in some crs mutants such as RIB-2 (crsC1) and GLN-2 (crsC2) were similar to that in the wild-type strain. Thus, it can be concluded that this enzyme may not be closely related to Crs^R phenotype.

The level of alkaline phosphatase among crs mutants fluctuated widely at the time of cell resuspension. It was also found that glucose completely inhibited the increase of this enzyme in some mutants but not in the others. Therefore, the level of this enzyme and changes in the enzyme ac-

tivity due to the presence of glucose may not be necessarily related to Crs^R phenotype.

Ohné (1975) has reported that malate dehydrogenase is repressed in cells grown in SP medium containing glucose. In the present study, however, it was found that the presence of glucose had little effect on the level of this enzyme in both the wild-type strain (WS) and the mutant strain (GLU-47). The difference between our results and Ohné's results (Ohné 1975) may be due to the media and the cultural conditions used. Our results suggest that malate dehydrogenase may not be involved in inhibition of sporulation of wild-type cells by glucose.

3. Effect of various inhibitors on sporulation

Previous studies have indicated that strains that carry crsA or crsF mutation are resistant not only to catabolites but also resistant to the inhibitory effect of novobiocin on sporulation (Takahashi and MacKenzie 1982). In the present study, sporulation of these mutants was found to be also resistant to cerulenin, alcohols and NaCl, agents that inhibited sporulation of wild-type strains. Since single mutation crsA or crsF alone causes resistance to all the agents tested, it is possible that the above agents affect sporulation through a common mechanism.

In E. coli, glucose is known to affect several membrane-related functions such as cell permeability, oxygen uptake (Halpern et al. 1964) and oxidative phosphorylation (Hempfling 1970). The amount of membrane-bound succinate dehydrogenase (Gray et al. 1966) and cytochromes (quoted in Haddock and Jones 1977) is reduced in the presence of glucose. Furthermoré, the amount of several outer membrane proteins is also altered by the presence of glucose or other carbon sources (Mallick and Herrlich 1979). Some of the above changes have been found to be due to a decrease in the intracellular concentration of cAMP. Glucose and other carbon sources are known to inhibit the activity of membrane-bound adenylate cyclase, the enzyme for the synthesis of cAMP (see review by Botsford 1981).

In B. subtilis, glucose is known to repress the TCA cycle enzymes such as citrate synthase (Flechtner and Hanson 1969) and aconitase (Ohné 1974). As in the case of E. coli, the synthesis of membrane-bound succinate dehydrogenase and cytochromes in B. subtilis is repressed by glucose (Kusaka 1973; Ohné 1975). Kusaka (1973) has reported that electrophoretic pattern of membrane proteins from cells of B. subtilis or B. cereus grown in the presence of glucose is very different from that in the absence of glucose. The same author has also found that the presence of glucose reduces contents of phospholipids and diglycerides. Moreover,

glucose represses induction of the gluconate-transport system in B. subtilis (Dowds et al. 1978). According to Rhaese and Groscurth (1976), phosphorylated metabolites of glucose suppress sporulation by inhibiting membrane-bound pppAppp synthetase.

Novobiocin is known to be an inhibitor of DNA gyrase (Gellert et al. 1976). However, Vazquez-Ramos and Mandelstam (1981) have found that this antibiotic does not inhibit DNA synthesis in sporulating cells. They conclude that the inhibitory effect of novobiocin on sporulation is not due to inhibition of DNA synthesis. These authors have found that although the synthesis of alkaline phosphatase is inhibited by novobiocin, serine protease synthesis is not affect by this antibiotic. They suggest that inhibition of transcription of certain genes may be the reason for the inhibitory effect of this antibiotic on spore formation. On the other hand, novobiocin has also been found to affect permeability of E. coli cells (Brock and Brock 1959). Hooper et al. (1982) have shown that novobiocin interacts not only with DNA gyrase but also with cellular components which are involved in the permeation of this antibiotic into E. coli cells. It is possible that novobiocin inhibits sporulation by affecting the membrane or membrane-associated functions.

Cerulenin has been known to be a specific inhibitor of fatty acid synthesis in both prokaryotic and eukaryotic

organisms (see review by Omura 1976). This antibiotic causes abnormal release of periplasmic β-lactamase in E. coli (Choudhurp 1978). It also inhibits secretion of various enzymes such as protease in B. subtilis (Mantsala 1982). Petit-Glatron and Chambert (1981) have found that the inhibition of synthesis of extracellular enzymes in B. subtilis by cerulenin is due to its physicochemical interaction with the membrane rather than its inhibitory effect on lipid synthesis. Wayne et al. (1981) have shown that Spo^{ts} phenotype induced by this antibiotic can be suppressed by the addition of various carbon sources or synthetic lipids into the culture media. These observations suggest that cerulenin probably inhibits sporulation by affecting the membrane.

The inhibitory effect of ethanol on sporulation has been investigated by Bohin and co-workers (Bohin et al. 1976b; Rigomier et al. 1980; Bohin and Lubochinsky 1982). These authors have shown that several sporulation-associated events, for example, excretion of extracellular enzymes are also inhibited by the presence of ethanol. Ethanol preferentially inhibits the synthesis of phosphatidylglycerol and markedly decreases the relative amount of branched fatty acids in phospholipids (Rigomier et al. 1980). According to these authors, the above changes may result in alterations in lipid-protein interactions and the expression of various membrane-associated functions. Bohin and Lubochinsky (1982)

have isolated ssa mutants which sporulate well in the presence of ethanol or other aliphatic alcohols. The composition of phopholipids in ssa mutants differs from that in wild-type strains. Bohin and Lubochinsky (1982) suggest that the regulation of membrane synthesis may be altered by the ssa mutation.

Ingram (1940) has reported that NaCl at concentrations of 0.6M or higher reduces the rate of respiration in B. cereus. This effect has been attributed to precipitation of some proteins in the membrane-bound respiratory chain by NaCl (Ingram 1947). In a marine bacterium, NaCl at 0.5M stimulates the activity of NADH oxidase, and at the same time it inhibits the activity of succinate dehydrogenase (Ichikawa et al. 1981). In addition, the compositions of phospholipids and fatty acids in a moderately halophilic bacterium Pseudomonas halosaccharolytica vary significantly in the presence of different concentrations of NaCl (Ohno et al. 1979). Alterations in the composition of phospholipids and fatty acids by NaCl have also been observed in a halotolerant bacterium, Staphylococcus aureus (Kanemasa et al. 1972). In E. coli, the presence of NaCl causes an increase in cyclopropane fatty acids and a decrease in unsaturated fatty acids in cells during the stationary phase (McGarrity and Armstrong 1975). As the melting points of cyclopropane fatty acids are higher than those of the homologous unsatu-

rated fatty acids, cyclopropanization of unsaturated fatty acids in the presence of NaCl will reduce membrane fluidity. A less fluid membrane will consequently affect various membrane-associated functions such as respiration and transport of substrates (McGarrity and Armstrong 1975). It appears that NaCl has a profound effect on the physicochemical state of the membrane and its associated functions.

The foregoing discussions on the mode of action of catabolites, novobiocin, cerulenin, ethanol and NaCl suggest that these agents inhibit sporulation by affecting the membrane structure or functions associated with the membrane. Indeed, several lines of evidence indicate the importance of the membrane or membrane-associated functions in the process of sporulation. It has been reported that activities of TCA cycle enzymes increase at the beginning of sporulation (Hanson et al. 1964; Ohné 1975). Activities in the electron transport system also increase significantly soon after t_0 (Taber et al. 1972; Felix and Lundgren 1973; Weber and Broadbent 1975). Mutants that fail to undergo these changes are asporogenous (Fortnagel and Freese 1968; Taber et al. 1972; Taber and Freese 1974). Changes in membrane protein composition during early stages of sporulation have been observed in both B. subtilis (Goldman 1976) and B. cereus (Andreoli et al. 1981). In addition, many spo0 mutants differ from wild-type strains in phospholipid compo-

sition and in various membrane-related functions (Ito et al. 1971; Schaeffer et al. 1971; Rigomier et al. 1974; Bohin et al. 1976a). Hence, it seems that certain alterations in the membrane take place during the initial stage of sporulation. If these alterations fail to occur or proceed improperly due to the presence of various inhibitors or spo0 mutations, sporulation will be blocked at early stages.

The effects of various membrane-affecting agents on sporulation of crs mutants are summarized in Table 31. With the exception of GLU-40, which may be affected in glucose transport or metabolism (Takahashi 1979), all the crs mutants are resistant to at least one of the membrane-affecting agents tested (Table 31). It is suggested that functions altered by these mutations may be related to the membrane. Indeed, our preliminary results of electrophoretic analysis of membrane proteins have revealed that in SP medium crsA mutant cells at $t_{-0.5}$ contain several membrane proteins which are practically absent in wild-type cells.

4. Suppressors of crs mutations

A suppressor mutation may occur in the gene that harbors the original mutation (intragenic suppressor); or, it may occur outside of the original mutant gene (intergenic suppressor). Intergenic suppressors can be divided into two

Table 31. Effect of various inhibitory agents on sporulation of crs mutants

Strain	Map position	Ethanol (0.7M)	Cerulenin (1 µg/mL)	Novobiocin (5 µg/mL)	NaCl (0.85M)
GLU-47 (crsA47)	225	R	R	R	R
RIB-2 (crsC1)	220	S	S	R	S
GLN-2 (crsC2)	220	S	R	R	S
MAN-A1 (crsD1)	5	S	S	S	R
MAN-B1 (crsE1)	10	R	R	S	R
MAL-4 (crsF4)	120	R	R	R	R
GLU-40 (crsB40)	55	S	S	S	S
WS (wild-type)	-	S	S	S	S

Note: R, resistant; S, sensitive. The data with novobiocin were reported by Takahashi and MacKenzie (1982).

catagories: direct suppressors and indirect suppressors. Direct suppressors achieve their suppressing effect by modifying tRNA or ribosomes so that mRNA transcribed from the original mutant gene can be translated into a protein which is functional or partially functional. Indirect suppressors, on the other hand, allow the expression of wild-type characters without altering the mutant gene products. Rather, they suppress the mutant phenotype by openning a new metabolic pathway, or removing the inhibitory molecules or modifying protein molecules (see review by Hartman and Roth 1973).

In the present study, the effect of various mutations which might act as suppressors for crs mutations was investigated. The effects of sca19, rfm11, eryl and relA mutations on the crs mutants are summarized in Table 32.

The sca mutations which were not linked to any of the crs mutations were found to be able to suppress Crs^R phenotype of crsA47 mutant. Thus, the sca mutations are acting as intergenic suppressors for crsA47. One of the sca mutations, sca19 can also suppress Crs^R phenotype of other crs mutants whose mutations are localized in various regions of the chromosome.

In E. coli, the inability of a mutant lacking the membrane-bound lactose permease to grow on lactose as the sole carbon source can be corrected by a number of suppressor

Table 32. Effect of various "suppressor mutations" on sporulation of crs mutants

crs mutation \ Suppressor mutation	scal9	rfml1	eryl	relA
crsA47	+	-	-	-
crsC1	+	+	+	-
crsD1	+	+	+	+
crsE1	+	+	+	-
crsF4	+	+	+	-

Note: +: suppressed (sensitive to glucose), -: not suppressed (resistant to glucose).

mutations that alter the cell envelope (Lazdunski and Shapiro 1972). In B. subtilis, it has been found that the abs mutation can suppress some of the membrane-associated phenotypes of a spo0A mutant by causing changes in the membrane proteins (Ito and Spizizen 1972). The present data show that the sca mutations suppress not only Crs^R phenotype of crsA47 mutant but also its resistance to a variety of membrane-affecting agents in sporulation. In our earlier discussion, the resistance to these agents has been suggested to be due to alterations in the membrane. It is possible that the sca mutations act as indirect suppressors which suppress the phenotypes of the crsA47 mutant by restoring the membrane to its wild-type state.

A rifamycin resistance mutation, rfm11 was shown to be able to suppress Crs^R phenotype of mutants that carried crsC1, crsC2, crsD1, crsE1 or crsF4. The rfm11 mutation did not inhibit sporulation of wild-type strains at both 37°C and 45°C, thus it represents a new type of rfm mutation which blocks sporulation of the crs mutants in the presence of glucose while allowing normal sporulation to take place in the absence of glucose.

Two hypotheses have been proposed to explain the mechanism by which RNA polymerase mutations block sporulation. According to one hypothesis, the mutations may interfere with the sequential modifications of RNA polymerase so

that the affected enzyme cannot transcribe sporulation-specific genes (Doi 1977; Losick 1982). The alternative hypothesis is that the affected RNA polymerase would change the rate of transcription and therefore create a metabolic imbalance which interferes with the process of sporulation (Wayne et al. 1981). Since rfm11 mutation did not affect sporulation in wild-type strains, the suppression of Crs^R phenotype by this mutation cannot be due to a failure to transcribe sporulation-specific genes. The rfm11 mutation probably acts as a suppressor for the crs mutants by creating a metabolic imbalance which occurs only in the presence of glucose.

An erythromycin resistance mutation, eryl could also suppress Crs^R phenotype of crsC1, crsD1, crsE1 and crsF4 mutants. This mutation has been shown to alter ribosomal protein L17 (Sharrock et al. 1981). Unlike rfm11, the eryl mutation causes Spo^{ts} phenotype in wild-type strains (Sharrock et al. 1981). The Spo^{ts} phenotype can be corrected by the addition of various carbohydrates or synthetic lipids to the culture media (Wayne and Leighton 1981). This observation has led these authors to conclude that eryl blocks sporulation of wild-type cells at high temperatures by causing a metabolic imbalance which disrupts the membrane. It is possible that eryl also suppresses Crs^R phenotype of the crs mutants by causing a metabolic imbalance. The present results

also show that our crsA47, crsE1 and crsF4 mutations can suppress the Spo^{ts} phenotype of eryl mutant. This observation suggests that the disruption in the membrane of eryl mutant at high temperatures can be compensated by these crs mutations.

In E. coli, the stringent response caused by amino acid starvation is known to affect the synthesis of rRNA, tRNA, ribosomal proteins and a number of other proteins. The synthesis of lipids is also under the stringent control (see review by Gallant 1979). As mutants of B. subtilis that carry relA mutation sporulate normally, it is suggested that the stringent response is not required for sporulation (Rhaese et al. 1975). In the present study, it was found that the relA mutation did not suppress sporulation of crsA47, crsC1, crsE1 and crsF4 mutants in the presence of glucose. However, Crs^{R} phenotype of crsD1 mutant was suppressed by the relA mutation. Thus, stringent response is required for Crs^{R} phenotype of the crsD1 mutant. The mechanism by which the stringent response is involved in achieving the Crs^{R} phenotype of crsD1 mutant is still unknown.

As the thorough characterization of the double mutants that carry crs mutations and suppressor mutations has not been carried out, this investigation is still in the preliminary stage. Further experiments, for example, electrophoretic analysis of membrane proteins from the double

mutants must be carried out before any definitive conclusion can be drawn.

In summary, a number of crs mutations which confer resistance to catabolites for sporulation have been mapped on the chromosome of B. subtilis. Among them, crsE1 and crsA47 mutations are located in genes for RNA polymerase, indicating that certain mutations in this enzyme can alter the response of cells to the inhibitory effect of catabolites on sporulation. Changes in the rates of growth and glucose utilization, and in the activities of IMP dehydrogenase, alkaline phosphatase and malate dehydrogenase are not necessary conditions to achieve catabolite resistance for sporulation. Sporulation of the crs mutants is resistant to at least one of the membrane-affecting agents, including novobiocin, cerulenin, ethanol and NaCl. The Crs^R phenotype and resistance to the membrane-affecting agents can be suppressed by several other mutations. It is suggested that functions affected in the crs mutants are related to the membrane, which is important in initiation of sporulation.

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