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New Mutation Affecting the Synthesis of Some Membrane Proteins and Sporulation in *Bacillus subtilis*

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A new mutation, *mpo*, which affects the synthesis of some membrane proteins and sporulation in *Bacillus subtilis* was identified. The *mpo* mutation was tightly linked to the overproduction of membrane proteins MP32 and MP18 (molecular weights of 32,000 and 18,000, respectively) and the temperature-sensitive sporulation phenotype. Genetic analysis showed that the *mpo* mutation maps between the *spoIIIB* and *lys* loci.

The sporulation of bacteria is a simple model system of unicellular differentiation, and the sporulation of *Bacillus subtilis* has been studied extensively (14). There are several reports describing the relationships between sporulation and the cell membrane. Ito and colleagues (8–10) showed that early-stage sporulation mutations, *spo0A* and *spo0B*, were reverted partially by either the *abs* or *tol* mutations, which affect membrane protein distribution. Goldman (4) showed that the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic pattern of membrane proteins of *B. subtilis* changes during sporulation. A relationship between changes in the cellular membrane and *B. subtilis* sporulation is also suggested by the studies of Bohin et al. (1, 2) and Guespin-Michel (5).

However, little is known about the role of membrane proteins in the *B. subtilis* sporulation process. To clarify this point, we attempted to isolate sporulation mutants which are defective in membrane proteins. We found that strain 1S48 (*spoIIIB*) overproduced several membrane proteins. However, genetic analysis showed that the overproduction of membrane proteins is not due to the *spoIIIB* mutation but due to a new mutation, *mpo*, located near the *spoIIIB* locus. In addition to the overproduction of several membrane proteins, we found that the *mpo* mutation is tightly linked to the temperature-sensitive sporulation phenotype. In this paper, we describe the identification and characterization of the *mpo* mutation.

MATERIALS AND METHODS

Bacterial strains and sporulation. *B. subtilis* strains used in this paper are shown in Table 1. Cells were grown in Schaeffer sporulation medium (16) unless otherwise indicated. Heat-resistant CFU were counted on nutrient agar (Difco Laboratories) plates after the cell suspension was heated for 10 min at 80°C.

Preparation of cell membranes. Cell membranes were prepared according to the method of Harmon and Taber (6). Cells from 50 ml of vegetative culture (Klett units 70 to 80 with filter no. 64) or from 10 to 20 ml of sporulating culture were harvested, washed twice by centrifugation and resuspension in 30 ml of 50 mM Tris-hydrochloride buffer (pH 7.6) containing 10 mM $MgCl_2$, 0.3 mM phenylmethylsulfonyl fluoride, and 5 mM magnesium-Titriplex (Merck), and suspended in 15 to 20 ml of 10 mM Tris-hydrochloride buffer (pH 7.0) containing 10 mM $(CH_3COO)_2Mg$, 100 mM KCl, 20% sucrose, 0.3 mM phenylmethylsulfonyl fluoride, and 5 mM magnesium-Titriplex. The cell suspension was treated with lysozyme (final concentration, 1 to 2 mg/ml) with mild shaking at 36°C for 1 to 2 h. Protoplasts were collected by centrifugation at 20,000 × *g* for 30 min and resuspended in 10 ml of 50 mM Tris-hydrochloride buffer (pH 7.2) containing 10 mM $MgCl_2$, 0.3 mM phenylmethylsulfonyl fluoride, 5 mM magnesium-Titriplex, 10 μg of RNase A (Sigma Chemical Co.) per ml, and 10 μg of DNase I (Sigma) per ml. After being kept on ice for 1 to 2 h, the lysates were centrifuged at 20,000 × *g* for 15 min. The membrane pellets were washed three times with deionized water by centrifugation at 20,000 × *g* for 15 min.

SDS-polyacrylamide gel electrophoresis of membrane proteins. Washed membrane pellets were resuspended in 150 to 300 μl of 100 mM Tris-hydrochloride buffer (pH 6.8) containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.3 mM phenylmethylsulfonyl fluoride, and 10 μg of bromophenol blue per ml and heated at 100°C for 3 min. Samples (20 to 30 μl) were applied onto SDS-polyacrylamide (12.5%) slab gels, and the electrophoresis was carried out with the discontinuous buffer system as described by Laemmli (11).

Two-dimensional gel electrophoresis of total cell proteins. Preparation of total cell proteins and two-dimensional gel electrophoresis were carried out as described by O'Farrell (12), except that Nonidet P-40 was replaced by Triton X-100, and 2% Ampholines in the pH range of 3.5 to 10 were used. Usually, cells from 30 ml of vegetative culture or 5 ml of sporulating culture were used.

Transformation. Transforming DNA was prepared by phenol extraction according to the method of Saito and Miura (15). Transformation was carried out at 36°C by the method of Bott and Wilson (3) with slight modification. The competent culture was grown in minimal medium supplemented with the mixture of nine amino acids (10 μg per ml each) described by Wilson and Bott (18), 50 μg of auxotrophic amino acids per ml, and 0.05% yeast extract. Two to three hours after the end of vegetative-growth phase, 1 ml of culture was mixed with DNA (1 μg per ml) and incubated for 30 min with shaking at 36°C. Then, 4 ml of Schaeffer sporulation medium was added, and the incubation was continued at 36°C for 24 h. The culture was heated at 80°C for 10 min, and after the appropriate dilution, 0.1 ml of the culture was spread on Schaeffer sporulation plates, which were then incubated at 36°C for 3 to 4 days. To assay for temperature-sensitive sporulation, colonies were picked up

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TABLE 1. *B. subtilis* strains

Strain	Genetic marker	Source and derivation
JH642	*pheA1 trpC2*	J. Hoch
1S9(JH646)	*pheA1 trpC2 spo0A12*	*Bacillus* Genetic Stock Center (BGSC), Ohio State University
1S16(JH648)	*pheA1 trpC2 spo0B136*	BGSC
1S17(JH647)	*pheA1 trpC2 spo0E11*	BGSC
1S19(JH649)	*pheA1 trpC2 spo0F221*	BGSC
14 UL	*spo0G*	Kyoto University
1S25	*trpC2 spo0H116*	BGSC
1S27	*metC3 tal-1 spo0J87*	BGSC
1S28	*trpC2 spo0K141*	BGSC
1S31	*trpC2 ade met spoIIA26*	BGSC
1S49	*trpC2 spoIIB131*	BGSC
1S43	*trpC2 spoIIC298*	BGSC
1S33	*trpC2 rpoB2 spoIID66*	BGSC
1S34	*trpC2 rpoB2 spoIIE61*	BGSC
1S59	*trpC2 spoIIF96*	BGSC
1S60	*leuA8 tal-1 spoIIG41*	BGSC
1S42	*metC3 tal-1 spoIIIA35*	BGSC
1S48	*trpC2 spoIIIB2 mpo*[a]	BGSC
HU5101	*trpC2*	Present paper; type I[a] transformant of 1S48
HU5201	*trpC2 mpo*[a]	Present paper; type II[a] transformant of 1S48
HLL-3g	*purB6 leuA8 metB5 hisA1 lys-21 thr-5 trpC2 nonA1*	Institute of Applied Microbiology, University of Tokyo

[a] For definition, see the text.

on sterilized tooth picks, transferred to a set of Schaeffer sporulation plates, and incubated for 3 to 4 days at 36 and 45°C, respectively.

Transduction. Transduction experiments were carried out by the method of Takahashi (17) by using PBS1 transducing phage.

RESULTS

SDS-PAGE pattern of JH642 membrane proteins. Before the analysis of membrane proteins of sporulation-defective mutants, the changes in the membrane protein pattern during sporulation of the wild-type strain JH642 were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Several new membrane proteins appeared during sporulation (Fig. 1a). Two of them, with molecular weights of 55,000 and 32,000, appeared at $T_{2.5}$ (2.5 h after vegetative-growth phase). Furthermore, when the membrane proteins were analyzed with a lower concentration (9%) of acrylamide gel, we found that one additional protein (molecular weight of 105,000) appeared at $T_{2.5}$. The pulse-chase experiment with the vegetative membrane proteins and the pulse-labeled experiment with the sporulating cell membrane proteins with a ^{14}C-amino acid mixture suggest that these newly appeared proteins are not the degradation products of the vegetative membrane proteins but are products newly synthesized during sporulation (data not shown). These proteins did not appear in most *spo0* mutants tested, suggesting that they may be associated with the sporulation process (manuscript in preparation).

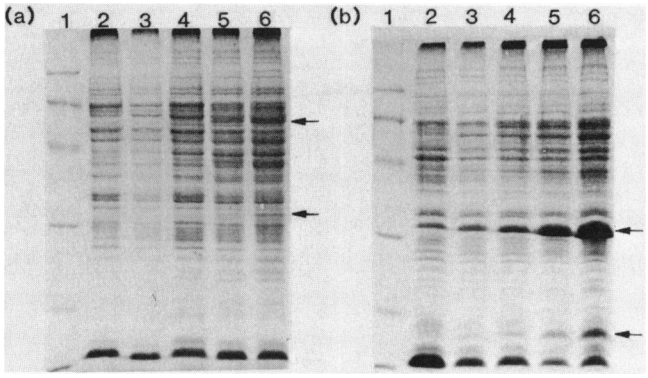

FIG. 1. SDS-polyacrylamide gel electrophoretic profiles of membrane proteins of strains JH642 (*spo*$^+$) and 1S48 (*spoIIIB*). Cells were grown at 36°C and harvested at intervals during vegetative-growth phase and sporulation or stationary phase. Preparation of membrane proteins and gel electrophoresis were carried out as described in the text. Samples (20 to 30 μl) were applied to SDS-polyacrylamide gels (12.5%). Arrows indicate the positions of new or overproduced proteins in each strain. (a) Strain JH642. Lane 1, molecular weight markers (phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soy bean trypsin inhibitor, 20,100; α-lactalbumin, 14,400); lane 2, vegetative-growth phase; lane 3, $T_{0.5}$; lane 4, $T_{1.5}$; lane 5, $T_{2.5}$; lane 6, $T_{3.5}$. (b) Strain 1S48. Lane 1, molecular weight markers; lane 2, vegetative-growth phase; lane 3, $T_{0.5}$; lane 4, $T_{1.5}$; lane 5, $T_{2.5}$; lane 6, $T_{3.5}$.

SDS-PAGE pattern of 1S48 membrane proteins. To search for sporulation mutants which have defects in membrane proteins, the membrane proteins of 17 sporulation mutants (8 *spo0* mutants, 7 *spoII* mutants, and 2 *spoIII* mutants; see Table 1) were examined by SDS-PAGE. One of them, strain 1S48, which has the *spoIIIB* mutation, showed overproduction of two membrane proteins, MP32 and MP18 (molecular weights of 32,000 and 18,000, respectively), during stationary phase (Fig. 1b). Two-dimensional gel electrophoresis of total cellular proteins showed that MP32 was different from

TABLE 2. Phenotypes of 1S48 Spo$^+$ transformants

Donor strain	Recipient strain	Selection	Type of transformant[a]		% Type I transformants
			I	II	
JH642 (*spo*$^+$)	1S48 (*spoIIIB*$^-$)	Spo$^+$	79	314	20.1

[a] Type I transformants formed raised, dark-brown colonies similar to those of strain JH642, whereas type II transformants formed flat, pale-brown colonies.

TABLE 3. Transformation experiments with HU5101 and HU5201 as donor strains and 1S48 as a recipient strain

Donor strain	Recipient strain	Selection	Type of transformant[a]		% Type I transformants
			I	II	
HU5101 (*spoIIIB*$^+$ *mpo*$^+$)	1S48 (*spoIIIB*$^-$ *mpo*$^-$)	Spo$^+$	59	156	27.4
HU5201 (*spoIIIB*$^+$ *mpo*$^-$)	1S48 (*spoIIIB*$^-$ *mpo*$^-$)	Spo$^+$	0	196	0

[a] For a description, see Table 2, footnote *a*.

the 32,000-dalton polypeptide of strain JH642 which appeared at $T_{2.5}$ (compare Fig. 2b and d).

Identification of the *mpo* mutation. To examine whether the *spoIIIB* mutation is the cause of the overproduction of MP32 and MP18, a transformation experiment was carried out with JH642 as a donor strain and 1S48 as a recipient strain, and Spo$^+$ transformants were selected. If the overproduction of MP32 and MP18 and the defect in sporulation are caused by a single mutation (*spoIIIB*), all Spo$^+$ transformants should form uniform colonies and should not overproduce MP32 and MP18. However, two types of Spo$^+$ transformants appeared (Table 2). Type I transformants (20 to 30% of the total Spo$^+$ transformants) formed raised, dark-brown colonies similar to those of strain JH642, whereas type II transformants formed flat, pale-brown colonies. This result suggests that in addition to the *spoIIIB* mutation, strain 1S48 may have a second mutation located near the *spoIIIB* locus. We designated this mutation as *mpo* (membrane protein overproduction).

To determine the genotype of strains JH642 and 1S48 with respect to the *mpo* mutation, transformation back-crosses were carried out by using HU5101 (type I transformant) and HU5201 (type II transformant) as donor strains and 1S48 as a recipient strain, selecting for Spo$^+$ transformants. The results are shown in Table 3. When HU5101 was used as a donor strain, two types of Spo$^+$ transformants (type I and type II) appeared. But when HU5201 was used as a donor strain, all Spo$^+$ transformants were type II. These results indicate that strain 1S48 has the same genotype (*mpo*$^-$) as strain HU5201, and strain JH642 has the same genotype (*mpo*$^+$) as strain HU5101 with regard to the *mpo* mutation.

To elucidate the relation between the overproduction of MP32 and MP18 and colony morphology, 12 colonies of type I transformants and 20 colonies of type II transformants were picked arbitrarily and grown in Schaeffer sporulation medium until $T_{4.5}$, and the overproduction of MP32 and MP18 was analyzed by SDS-PAGE. The results are shown in Table 4. The overproduction of MP32 and MP18 was not observed in any type I transformants but was observed in all type II transformants. These results suggest that the overproduction of MP32 and MP18 is tightly linked to the type II phenotype.

To know whether the *mpo* mutation affects the synthesis of cytoplasmic proteins, total cellular proteins of strains HU5101 (*mpo*$^+$) and HU5201 (*mpo*$^-$) obtained from the vegetative-growth phase and sporulation phase ($T_{4.5}$) were analyzed by two-dimensional gel electrophoresis. When the total cellular protein pattern of both strains was analyzed according to the method of O'Farrell (with 2% Ampholines comprised of 1.6% in the pH range of 5 to 7 and 0.4% in the pH range of 3.5 to 10), no significant differences were found between strains HU5101 and HU5201, except in the case of MP18 (data not shown). However, when the patterns of the total cellular proteins were analyzed by the modified system with 2% Ampholines in the pH range of 3.5 to 10 only, at least one cytoplasmic protein (CP23, molecular weight of 23,000) was found to be overproduced at $T_{4.5}$ in strain HU5201 (Fig. 2). This result suggests that the *mpo* mutation

TABLE 4. Relationship between the type of Spo$^+$ transformants and the overproduction of MP32 and MP18

Type of transformant	No. of colonies tested[a]	No. of colonies containing overproduced membrane proteins[b]:	
		MP32	MP18
I	12	0	0
II	20	20	20

[a] The type I and type II colonies were arbitrarily selected from the Spo$^+$ transformants of the experiment described in Table 2.

[b] The preparation of membrane proteins was carried out as described in the text. Cells were incubated at 36°C until $T_{4.5}$ and then harvested.

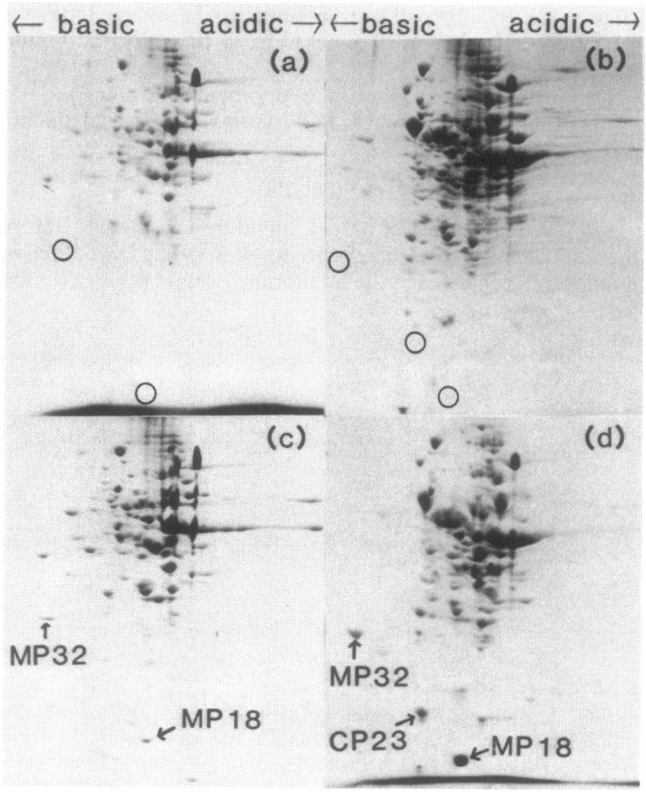

FIG. 2. Two-dimensional electrophoretic profiles of total cellular proteins of strains HU5101 (*mpo*$^+$) and HU5201 (*mpo*$^-$). Preparation of total cellular protein and two-dimensional gel electrophoresis were carried out as described in the text. Cells were grown at 36°C and harvested at vegetative-growth phase and $T_{4.5}$. Samples (100 μl) were applied to the SDS-polyacrylamide gel (12.5%). Arrows indicate the positions of the overproduced proteins in the mutant strain. Circles indicate the positions where the overproduced proteins are found in the mutant strain. (a) Strain HU5101, vegetative-growth phase; (b) strain HU5101, $T_{4.5}$; (c) strain HU5201, vegetative-growth phase; (d) strain HU5201, $T_{4.5}$.

TABLE 5. Mapping of *mpo* in relation to the *spoIIIB* and *lys* loci[a]

Donor strain	Recipient strain	Selection	Recombinant class			No. of recombinants
			spoIIIB	*mpo*[b]	*lys*	
1S48	HLL-3g	Lys$^+$	1	1	1	20
			1	0	1	
			0	1	1	12
			0	0	1	48
HLL-3g	1S48	Spo$^+$	1	1	1	37
			1	0	1	0
			1	1	0	2
			1	0	0	14

[a] 1 and 0 refer to donor and recipient phenotypes, respectively. The possible order of the loci is *spoIIIB mpo lys*.
[b] The *com*$^-$ transductants form flat, pale-brown colonies which sporulate at 36°C but sporulate poorly at 45°C.

affects not only the synthesis of membrane proteins but also the synthesis of at least one cytoplasmic protein.

Genetic mapping of *mpo* mutation. Genetic analysis (Table 2) showed that the *mpo* mutation was located near the *spoIIIB* locus. Therefore, we tried to map the *mpo* locus in relation to the *spoIIIB* and *lys* loci. The *lys* locus is near the *spoIIIB* locus (7). Since all type II transformants shown in Table 3 showed temperature-sensitive sporulation on Schaeffer sporulation plate at 45°C (see also Fig. 4), the temperature-sensitive sporulation phenotype was used as a marker for the *mpo* mutation. At first, three-factor transduction crosses were carried out by using 1S48 as a donor strain and HLL-3g as a recipient strain, and Lys$^+$ transductants were selected. The results are shown in Table 5. Recombinants with both mutations *spoIIIB* and *mpo* were phenotypically indistinguishable from those having *spoIIIB* alone. If the *mpo* mutation lies to the left of *spoIIIB-lys*, the 0-1-1 class should be low since this class is generated by multiple crossovers. However, since the 0-1-1 class is relatively high, we thought that the *mpo* mutation might lie between the *spoIIIB* and *lys* loci. To confirm this order, we tried the

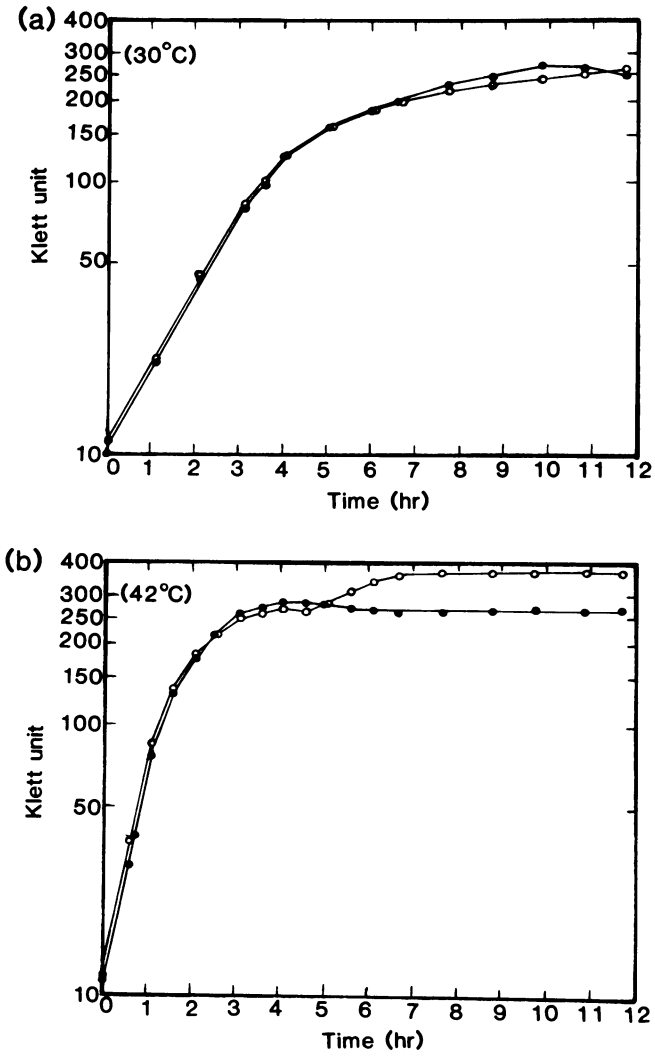

FIG. 3. Growth curves of strains HU5101 (*mpo*$^+$) and HU5201 (*mpo*$^-$). HU5101 and HU5201 were grown in Schaeffer sporulation medium with shaking at (a) 30°C and (b) 42°C, respectively. Symbols: ○, HU5101 (*mpo*$^+$ *trpC*$^-$); ●, HU5201 (*mpo*$^-$ *trpC*$^-$).

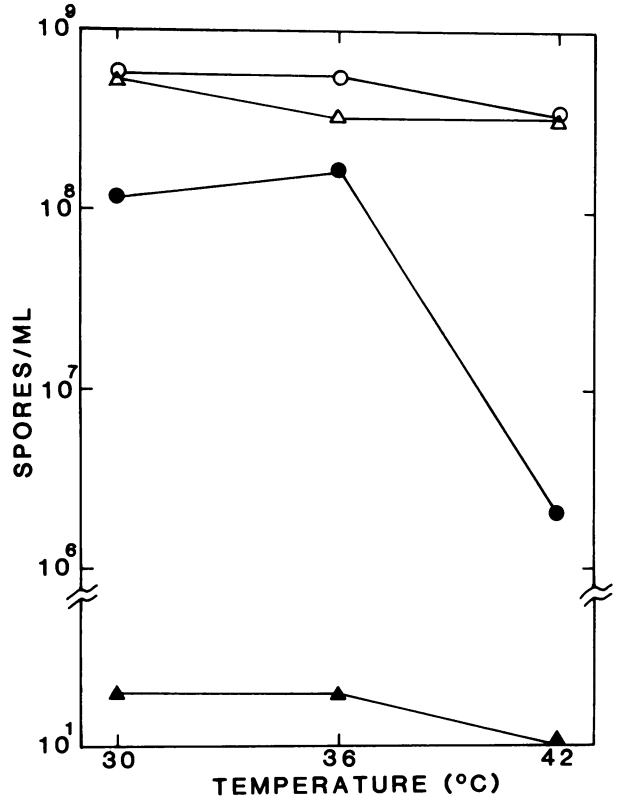

FIG. 4. Sporulation of strains JH642, 1S48, HU5101, and HU5201. Sporulation was determined after 48 h of incubation in Schaeffer sporulation medium at 30, 36, and 42°C, respectively. Symbols: △, JH642; ▲, 1S48; ○, HU5101; ●, HU5201.

reciprocal transduction with HLL-3g as a donor strain and 1S48 as a recipient strain, selecting for Spo^+ transductants. The results (Table 5) show that the *mpo* mutation lies between the *spoIIIB* and *lys* loci. There is a possibility that the *mpo* mutation might have been introduced into strain 1S48 together with the *spoIIIB* mutation when the strain was isolated from the wild-type strain by acridine orange mutagenesis (13).

Growth and sporulation of HU5101 (*mpo*$^+$) and HU5201 (*com*$^-$). To characterize the *mpo* mutation, comparison of strains HU5101 (mpo^+) and HU5201 (mpo^-) was carried out, since the genetic background is identical in both strains except that HU5101 is mpo^+ but HU5201 is mpo^-. The growth rate of both strains is the same in Schaeffer sporulation medium at 30 and 42°C, respectively (Fig. 3), suggesting that the *mpo* mutation does not affect vegetative growth. However, the *mpo* mutation is tightly linked to temperature-sensitive sporulation phenotype (Fig. 4). The level of sporulation of strain HU5201 is slightly lower than that of strain HU5101 at 30 and 36°C, but when strain HU5201 is incubated at 42°C, the sporulation frequency decreased to about 1/100 of that observed at permissive temperature.

DISCUSSION

The present paper describes a new mutation, *mpo*, which affects the synthesis of several proteins and the sporulation ability of *B. subtilis*. The *mpo* mutation did not affect vegetative growth but increased the synthesis of two membrane proteins, MP32 and MP18 (molecular weights of 32,000 and 18,000, respectively), and was tightly linked to the temperature-sensitive sporulation phenotype. Furthermore, two-dimensional gel electrophoresis of total cellular protein showed that in addition to MP32 and MP18, the *mpo* mutation stimulated the synthesis of at least one cytoplasmic protein during sporulation time periods.

Goldman (4) has described membrane protein alterations during the early stages of sporulation in *B. subtilis* wild-type cells, as well as in an asporogenous mutant (*spoIVC*). We observed that several new membrane proteins with molecular weights of 105,000, 55,000, and 32,000 appeared and that at least one protein (molecular weight of 33,000) disappeared during sporulation in wild-type strains. However, we found that the 105,000-, 55,000-, and 32,000-dalton polypeptides did not appear during stationary phase in most *spo0* mutants of *B. subtilis* (manuscript in preparation), suggesting that these membrane proteins are associated with sporulation.

It is still uncertain how the *mpo* mutation is tightly linked to the temperature-sensitive phenotype. Since the overproduction of MP32 and MP18 was observed in strain HU5201 (mpo^-) grown at 36°C but sporulation was almost normal, the overproduction of MP32 and MP18 in the *mpo* mutant may not be a direct cause of defective sporulation. However, since these membrane proteins were also overproduced at 42°C (data not shown), the overproduced membrane proteins may disturb sporulation at the nonpermissive temperature.

There are several reports indicating that sporulation mutants have membrane defects. For example, Bohin et al. (1) showed that several *spo0* mutants were hyperproducers of membrane-bound nitrate reductase A. By the analysis of partial revertants of *spo0A* and *spo0B* mutants, Ito et al. (8–10) suggested that *spo0A* and *spo0B* mutants have defective membranes. The *mpo* mutation may decrease sporulation at nonpermissive temperatures by affecting the structure of the membrane due to the overproduction of MP32 and MP18. Further genetic and biochemical studies will clarify how the *mpo* mutation causes the overproduction of several proteins and affects sporulation.

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