A Suppressor of Nonsense Mutations in *Bacillus subtilis*

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The suppressor-sensitive mutants of phage φ29, susB47 and susN212, do not induce the synthesis of proteins NP1 and II, respectively, when they infect *Bacillus subtilis* sus-. A new polypeptide of molecular weight 75000, smaller than that of protein NP1, is produced in susB47-infected cells. An analysis of the methionine-containing tryptic peptides of protein NP1 and the 75000-molecular-weight polypeptide showed that both proteins are related. When mutants susB47 and susN212 infect *B. subtilis* su+3, a small amount of proteins NP1 and II, respectively, is synthesized, the level of the suppression being about 10%. The results indicate that the *B. subtilis* su+3 strain is a suppressor of nonsense mutations.

Nonsense mutants are very useful to detect protein gene products as under restrictive conditions a complete protein is not synthesized; instead, a fragment of length related to the position of the mutation is produced [1]. In *Escherichia coli* several strains which suppress the nonsense codons are available [2-4].

More recently several suppressor strains from *Bacillus subtilis* have been isolated [5-7] but there is no direct evidence as to the nature of the suppressor. By using asporogenous derivatives of the *B. subtilis* strain Mu8u5u5 su+3 isolated by Georgopoulos [6], two collections of suppressor-sensitive mutants of bacteriophage φ29 have been isolated [8,9]. In this paper we show that a sus mutant of φ29 produces, in *B. subtilis* su+, a fragment of the normal protein. This mutation, as well as others, are partially suppressed when the mutants are grown in the suf3 strain of *B. subtilis,* giving rise to the appearance of a small amount of the normal phage proteins. These results indicate that the *B. subtilis* su+3 strain [6] contains a suppressor of nonsense mutations.

MATERIALS AND METHODS

Bacteria and Phage

The non-permissive host *B. subtilis* 168 try- spoOA-110NA [10] was obtained from Dr P. Schaeffer. The permissive host, *B. subtilis* 168 MO-99 [met- thr+] su+3 spoOA-3NA, was prepared [9] by transformation

**Enzyme.** Pancreatic ribonuclease (EC 2.7.7.17).

Ultraviolet Irradiation and Labelling of Bacteria

The bacteria were grown in minimal medium and irradiated with ultraviolet light for 6 or 7.5 min prior to infection as described by Carrascosa et al. [12]. The irradiated bacteria were resuspended, at $5 \times 10^8$ cells/ml, in minimal medium containing 0.5 mM amino acids except leucine which was 0.01 mM. Aliquots of the irradiated bacteria were infected with wild-type phage or with the mutants susB47 or susN212 at a multiplicity of 20 and shaken at 37 °C. The bacteria infected with the sus mutants were labelled with $[^{3}H]$leucine (25 μCi/ml; 0.023 mM) from 20 to 23 min after infection; at 23 min a 100-fold excess of non-radioactive leucine was added and the incubation was continued for 2 min. Wild-type infected and uninfected bacteria were pulse-labelled at the same times with $[^{14}C]$-leucine (7.5 μCi/ml; 0.023 mM) and chased with non-radioactive leucine for 2 min. To follow phage development aliquots were taken from the infected cultures at different times and plated on *B. subtilis* MO-99 su+3. The labelled bacteria were resuspended in half the original volume of a buffer containing 1 mM EDTA, 0.58 mM phenylmethylsulfonyl fluoride and either
10 mM sodium phosphate, pH 7.2, or 30 mM Tris-HCl, pH 6.8, depending on whether a continuous or a discontinuous pH system of electrophoresis was used, and incubated with lysozyme (280μg/ml) for 2.5 h at 4°C. Then, the sample was frozen and thawed three times and treated with pancreatic ribonuclease (10μg/ml) for 30 min at 0°C. To eliminate residual radioactive amino acid the lysed cells were treated at 4°C with 100% trichloroacetic acid to give a final concentration of 5%; after 15 min in ice, the precipitate was centrifuged and treated with pancreatic ribonuclease as described in each case. The gels were cut in 0.8–1.0-mm slices with a Mickle gel slicer and the radioactivity determined as described [12].

**Tryptic-Peptide Analysis**

Cells labelled as described before were lysed and subjected to polyacrylamide gel electrophoresis using a discontinuous pH system [14]. The gels were cut in 0.8-mm slices and the protein was eluted of each slice by overnight incubation at 37°C in 0.5 ml of 0.1% sodium dodecylsulfate and 0.58 mM phenylmethylsulfonyl fluoride after freezing and thawing three times. A 25-μl aliquot of each fraction was counted and the peak fractions of the proteins to be analyzed were pooled, lyophilized and the residue was dissolved in 1 ml of water. Electrophoresis of proteins from uninfected cells, labelled in the same conditions as the infected cells, showed that the radioactivity present in the position corresponding to the protein peaks to be subjected to tryptic digestion was negligible.

A mixture of 85S-labelled and 3H-labelled proteins was reduced, carboxymethylated and digested with trypsin after removing the dodecylsulfate as described elsewhere [15]. The tryptic digest was applied to a column (22×0.9 cm) of Beckman PA-35 ion-exchange resin maintained at 50°C. The column was developed under pressure, at a rate of about 1 ml/min, with a linear gradient of 200 ml of 0.2 M pyridine—acetic acid (pH 3.1) and 200 ml of 2 M pyridine—acetic acid (pH 5.0). Fractions of 2 ml were collected in small vials (4.5×1.2 cm) containing a disk of glass-fiber paper (Whatman GF/A, 2.4 cm diameter), dried at 90°C and counted as described [16].

**Polyacrylamide-Gel Electrophoresis**

Polyacrylamide gel electrophoresis was carried out either in a continuous pH system on 15-cm-long gels containing 12.5% acrylamide, 0.6% N,N’-methylenebisacrylamide, 8 M urea and 0.1% sodium dodecylsulfate [13] or, for a better separation of the structural proteins, in a discontinuous pH system on 10-cm-long gels containing 10% acrylamide, 0.25% N,N’-methylenebisacrylamide and 0.1% sodium dodecylsulfate [14].

The samples for electrophoresis were dissociated in a buffer containing either 5 mM sodium phosphate, pH 7.1, 1% (w/v) sodium dodecylsulfate, 1% (v/v) 2-mercaptoethanol and 8 M urea or 0.0625 M Tris-HCl pH 6.8, 2% sodium dodecylsulfate, 5% 2-mercaptoethanol and 6 M urea, depending on whether the continuous or discontinuous pH system of electrophoresis was used. The samples were heated for 5 min in a bath of boiling water. Electrophoresis was run as described in each case. The gels were cut in 0.8–1.0-mm slices with a Mickle gel slicer and the radioactivity determined as described [12].

**RESULTS**

**Proteins Induced after Infection of B. subtilis su- with sus Mutants of Phage φ29**

Fig.1A shows no phage development after infection of B. subtilis 110NA su- with mutants susB47 or susN212. The proteins synthesized after infection with these mutants were labelled with [3H]leucine in a 3-min pulse from 20 to 23 min after infection followed by a 2-min chase, and analyzed by gel electrophoresis in the presence of sodium dodecylsulfate and urea. As previously shown [12] wild-type phage φ29 (Fig.2A)
the synthesis of the structural proteins NP1 (neck appendages), TP1 (tail), HPI (head major protein), NP2 (upper collar), and NP3 (lower collar) (not resolved in this experiment), and HP3 (head fibers), as well as that of the non-structural proteins II—XII. Protein I is not seen in this gel as it moves at about the same position as protein HP3, which at the time of the pulse is synthesized in larger amount; protein IV is also not seen in this pulse as it is a minor protein whose synthesis is maximal at late times after infection [12]. Mutant susB47 induces the synthesis of all the proteins present in wild-type infected cells except that of protein NP1 (Fig. 2A and B). The proteins labelled in uninfected cells are shown in Fig. 2B.

The absence of protein NP1 in susB47-infected cells is more clearly seen when the electrophoresis is carried out using a discontinuous pH system. As shown in Fig. 3A, no protein NP1 (Mr = 80000) appears and, instead, a new polypeptide (Mr = 75000), with mobility intermediate between that of proteins NP1 and TP1, is present in the mutant-infected cells. This polypeptide is absent from uninfected cells (Fig. 3B). When the phage-specific radioactivity is calculated by the method of Mayo and Sinsheimer [17] it is clearly seen that the polypeptide of molecular weight 75000 is specific to mutant-infected cells, and protein NP1 is absent (Fig. 3C). This result suggests that the new polypeptide is a fragment of protein NP1. Mutant susN212 induces, in B. subtilis su−, the synthesis of all the proteins seen after infection with wild-type phage except protein II, a late non-structural protein (Fig. 4A). As seen in Fig. 4B and C, no phage-specific radioactivity is present in the position of protein II.

**Suppression of sus Mutations in B. subtilis su+3**

When mutants susB47 or susN212 infect B. subtilis MO-99 su+3 (Fig. 1B), phage development takes place, in contrast with what happens after infection of the su− strain (Fig. 1A).

Fig. 3 (D—F) shows the proteins synthesized in B. subtilis su+3 infected with mutant susB47. In this case, there is a small amount of protein NP1, which
Nonsense Suppressor in B. subtilis

Fig. 3. Gel electrophoresis in a discontinuous pH system of the proteins induced by infection of ultraviolet-irradiated B. subtilis su- or su+ with mutant susB47. Cells were irradiated, infected with mutant susB47 or wild-type phage and labelled as described in Fig. 2 except that irradiation of B. subtilis su+ was for 6 min and that of B. subtilis su- was for 7.5 min. Cells infected with mutant susB47 were labelled with [3H]leucine; cells infected with wild-type phage and uninfected cells were labelled with [14C]leucine. Electrophoresis using a discontinuous pH system [14] was carried out at a constant voltage of 90 V for 6.5 h. (A) Co-electrophoresis of susB47-infected B. subtilis su- (●—●) and wild-type infected su- (O—O). (B) susB47-infected su- (●—●) and uninfected su- (O—O). (C) susB47-specific radioactivity in su- bacteria calculated according to the method of Mayol and Sinsheimer [17]. (D) susB47-infected B. subtilis su+ (●—●) and wild-type infected su+ (O—O). (E) susB47 infected su- (●—●) and uninfected su+ (O—O). (F) susB47-specific radioactivity in su+ bacteria calculated according to the method of Mayol and Sinsheimer [17].

overlaps with that present in wild-type infected cells, contrary to the complete absence of this protein when su- bacteria were infected with the same mutant (Fig. 3A—C). This protein is phage-specific as shown by the lack of a corresponding peak in uninfected cells (Fig. 3E and F). By addition of the radioactivity present in protein NP1 and the 75000-molecular-weight polypeptide in susB47-infected cells (Fig. 3D), a suppression of about 12% can be calculated.

Fig. 4 (D—F) shows the presence of a small peak of protein II which is phage-specific (see Fig. 4F), after infection of B. subtilis su+ with mutant susN212. From the ratio of protein HP3 to protein II in wild-type infected cells and that of these proteins in susN212-infected cells a suppression of about 8% can be estimated, a value similar to that obtained for mutant susB47.

Relationship between Protein NP1 and the 75000-Molecular-Weight Protein Produced in susB47-Infected B. subtilis su-

To study whether or not the polypeptide of molecular weight 75000 produced in susB47-infected B. subtilis su- is a fragment of protein NP1, the methionine-containing tryptic peptides of both proteins were analyzed as described in Materials and Methods. As shown in Fig. 5A both proteins contain very similar peptides indicating a relationship between them.

Fig. 4. Polyacrylamide-gel electrophoresis of the proteins induced by injection of ultraviolet-irradiated B. subtilis su- or su+ with mutant susN212. Cells were irradiated, infected with mutant susN212 or with wild-type phage and labelled as described in Fig. 2 and 3. Electrophoresis was carried out as described in Fig. 2. The recovery of radioactivity ranged between 80 and 95%. (A) Coelectrophoresis of susN212-infected B. subtilis su- (●—●) and wild-type infected su- (O—O). (B) susN212-infected su+ (●—●) and uninfected su- (O—O). (C) susN212-specific radioactivity in su- bacteria calculated according to Mayol and Sinsheimer [17]. (D) susN212-infected B. subtilis su+ (●—●) and wild-type infected su- (O—O). (E) susN212 infected su+ (●—●) and uninfected su+ (O—O). (F) susN212-specific radioactivity in su+ bacteria calculated according to the method of Mayol and Sinsheimer [17].

It has been suggested that protein NP1 is synthesized as a precursor of higher molecular weight [18]. By pulse-chase experiments and tryptic peptide analysis we have confirmed this possibility and shown the existence of a precursor of protein NP1 (P-NP1) with a molecular weight value of about 90000 (unpublished results). This protein can be seen in Fig. 4 as a peak with a mobility slightly lower than that of protein NP1. To study the relationship between P-NP1 obtained from wild-type infected cells and the fragment produced in susB47-infected cells, the methionine-containing tryptic peptides of both polypeptides were studied. Fig. 5B shows the similarity in the peptides obtained from both proteins. On the other hand, by comparing the methionine-containing tryptic peptides of protein NP1 and P-NP1, it can be seen that they are essentially identical (Fig. 5A and B).

DISCUSSION

To study whether or not the suppressor strain from B. subtilis sus+ isolated by Georgopoulos [6] is a suppressor of nonsense mutations, the proteins induced after infection of the B. subtilis su- strain by suppressor-sensitive mutants of phage φ29 were analyzed by polyacrylamide gel electrophoresis. A nonsense mutant would give rise to the disappearance of a protein and the corresponding appearance of a fragment of a size dependent on the position of the mutation. On the other hand, a missense mutation
would produce a protein with the same molecular weight as the normal protein, and the same electrophoretic mobility in the presence of sodium dodecyl-sulfate.

When *B. subtilis* su− was infected with the φ29 mutants susB47 and susN212 the main phage-induced proteins were synthesized except proteins NP1 and II, respectively, suggesting that these mutants are nonsense. Moreover, a fragment of molecular weight 75000 was synthesized after infection with mutant susB47 instead of protein NP1 (molecular weight 80000). On the other hand, infection with the temperature-sensitive. mutant tsB73 [11] under restrictive conditions gave rise to the synthesis of proteins P-NP1 and NP1 (unpublished results), as would be expected for a missence mutation. No fragment was seen after infection with mutant susN212. It is possible that the fragment produced by this mutant is of small molecular weight and runs out of the gel, that it overlaps with other phage-induced proteins or that it is degraded as has been shown in other cases [19]. sus mutants in the genes coding for the φ29 appendage protein and for protein II have also been characterized by Anderson and Reilly as the products of genes J and N, respectively [18].

Tryptic peptide analysis of the fragment of molecular weight 75000 which appears in susB47-infected *B. subtilis* su− cells showed that the methionine-containing peptides of this protein and those of NP1 are very similar. Moreover, the tryptic peptides of the fragment are also very similar to those of P-NPI, a precursor of protein NP1.

When mutants susB47 and susN212 of φ29 were grown in the *B. subtilis* strain MO-99 su+3, a small, but significant amount of proteins NP1 and II was observed, respectively. The level of suppression of the mutation was about 10%.

These results indicate that the suppressor strain su+3 isolated by Georgopoulos [6] is a suppressor of nonsense mutations. This is in agreement with the existence of a polar effect in sus mutants of phage φ29 belonging to two adjacent cistrons, E and H [9].

Fig. 5. Tryptic peptide analysis of proteins NP1 and P-NP1 and the 75000-molecular-weight polypeptide synthesized in susB47-infected *B. subtilis* su−. 35S-labelled protein NP1 or 3H-labelled P-NP1 produced in wild-type infected cells, and 3H-labelled 75000-molecular-weight polypeptide synthesized in susB47-infected *B. subtilis* su− were isolated as described in Materials and Methods. The mixtures indicated below were reduced, carboxymethylated, treated with trypsin and chromatographed through a column of Beckman PA-35 ion-exchange resin as indicated. The recovery was about 80%. (A) Tryptic peptides of a mixture of 13000 counts 35S-labelled NP1/min (O-----O) and 30000 counts 3H-labelled 75000-molecular-weight polypeptide/min (●●●●). (B) Tryptic peptides of 18000 counts 35S-labelled P-NP1/min (O-----O) and 30000 counts 3H-labelled 75000-molecular-weight polypeptide/min (●●●●).
However, the suppressor seems to be a weak one, suggesting that we may be dealing with an ochre suppressor. Mutants in phage genes coding for proteins needed in large amounts may not be easy to isolate using a weak suppressor. To solve this problem, stronger nonsense suppressors of *B. subtilis* are being isolated.

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