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Isolation of a Strong Suppressor of Nonsense Mutations in *Bacillus subtilis*

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By treatment of *Bacillus subtilis* MO-101-P *spoA*$^-$ *met thr*$^-$ *su*$^-$ with ethyl methanesulfonate, a strong suppressor strain of nonsense mutations, *B. subtilis* MO-101-P *spoA*$^-$ *[met*$^-$*]*$^+$ *thr*$^-$ *su*$^{+44}$, was isolated. This strain does not suppress phage Φ29 mutant *susB*47, selected on a *B. subtilis* strain containing the *su*$^{+3}$ suppressor isolated by Georgopoulos. A revertant from this mutant, *susB*610, was isolated, being suppressed by both the *su*$^{+3}$ and *su*$^{+44}$ suppressor strains. The efficiency of suppression by strain *su*$^{+44}$ is about 50%. The experiments shown in this paper suggest that strain *su*$^{+44}$ contains an amber and strain *su*$^{+3}$ an ochre suppressor.

In *Escherichia coli*, several suppressors of nonsense mutations are known. Amber and opal suppressors are rather strong (20–60% chain propagation) whereas ochre suppressors are fairly weak (about 5–10% chain propagation). It has been shown that triplets UAA (ochre) and UGA (opal) can only be recognized by ochre and opal suppressors, respectively, whereas the triplet UAG (amber) can be recognized by both amber and ochre suppressors [1–4].

Whether or not a mutant is effectively corrected by a suppressor depends, in addition, on the acceptability of the amino acid provided by the suppressor tRNA and on the amount of gene product required. Amber mutants affecting catalytic genes are suppressed, at least by some ochre suppressors, but generally, mutants affecting stoichiometric genes are not [5, 6]. Thus, nonsense mutants isolated on an ochre suppressor may be ochre or amber mutants, mainly from catalytic genes, whereas nonsense mutants isolated on an amber suppressor are ambers, from catalytic or stoichiometric genes.

Several suppressor strains have been isolated in *Bacillus subtilis* [7–9]. Two of these strains, *B. subtilis* Mu8u5u5 *su*$^{+3}$ [8] and *B. subtilis* HA101B [7] were characterized by Camacho *et al.* [10] and by Shub [11], respectively, as suppressors of nonsense mutations.

The suppression efficiency of the Georgopoulos *su*$^{+3}$ strain from *B. subtilis* is only about 10% [10], suggesting that it may be an ochre suppressor. Two collections of suppressor-sensitive (*sus*) mutants of phage Φ29 have been isolated with such a suppressor [12, 13]. If *su*$^{+3}$ is indeed an ochre suppressor and if the situation is similar to that found in *E. coli*, these *sus* mutants are presumably ambers or ochres affecting mainly catalytic genes; mutants in genes coding for proteins needed in large amounts, like some major structural proteins and other stoichiometric proteins, might not be easy to isolate.

To overcome that possibility we tried to isolate a stronger suppressor from *B. subtilis*, starting with a *spoA*$^-$ *leu*$^+$ derivative of strain Mu8u5u5 *leu*$^-$ *met*$^-$ *thr*$^-$ *su*$^-$ [8]. In strain *su*$^{+3}$, the methionine and threonine requirements are suppressed; if strain *su*$^{+3}$ is indeed an ochre suppressor, either or both of these mutations may be amber, and this would permit isolation from the *su*$^-$ strain of amber suppressor derivatives, which would suppress either or both mutations.

In this paper we report the isolation of a strong suppressor strain, *B. subtilis [met*$^-$*]*$^+$ *thr*$^-$ *su*$^{+44}$, with an efficiency of suppression of about 50%. The evidence presented suggests that it may be an amber suppressor.

MATERIALS AND METHODS

Bacteria, Phage and Media

The nonpermissive host, *Bacillus subtilis* 168 MO-101-P *met*$^-$ *thr*$^-$ *spoA*$^-$ *su*$^-$, was prepared by Dr F. Moreno by transformation of *B. subtilis* Mu8u5u5

Eznymes. Pancreatic ribonuclease (EC 3.1.4.22); lysozyme or mucopeptide *N*-acetylmuramylhydrolase (EC 3.2.1.17).

leu$^-$ *met*$^-$ *thr*$^-$ *su*$^-$ with DNA from *B. subtilis* Marburg 3NA *spoA*$^-$ *su*$^-$. *B. subtilis* 110NA *try*$^-$ *spoA*$^-$ *su*$^-$ was obtained from Dr P. Schaeffer. The permissive host *B. subtilis* 168 MO-99 [*met*$^-$ *thr*$^-$]$^+$ *spoA*$^-$ *su*$^{+3}$, containing the suppressor isolated by Georgopoulos [8], was prepared as described [13].

Most of the suppressor-sensitive (*sus*) mutants used were from the collection of Moreno *et al.* [13]. Mutants *susG*22, *susD*121 and *susD*172 were produced by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and scored on *B. subtilis* MO-101-P *thr*$^-$ [*met*$^-$]$^+$ *spoA*$^-$ *su*$^{+44}$, obtained as described in this paper.

Mutant *susB*610, a revertant from *susB*47 [13], was isolated by its ability to grow on strains *su*$^{+44}$ and *su*$^{+3}$. The original mutant, *susB*47, was able to grow on strain *su*$^{+3}$ but not on *su*$^{+44}$.

The preparation of phage stocks and the phage assays were as described [14].

Minimal medium [15], broth [13] and phage buffer [16] were as described.

Mutagenesis and Selection of Mutants

B. subtilis MO-101-P *su*$^-$ grown in minimal medium until a cell concentration of 10^8/ml, was concentrated 2-fold by centrifugation and resuspension in the same medium. An aliquot was diluted 4-fold in 1 M Tris-HCl pH 7.5 in the presence of 4% (v/v) ethyl methanesulfonate [17]. The mixture was shaken strongly at 26 °C and after 1 and 2 min 2-ml aliquots were removed, filtered through nitrocellulose filters and washed three times with 1 M Tris-HCl, pH 7.5, to remove the ethyl methanesulfonate. The cells were resuspended in broth and aliquots of the culture were allowed to segregate till they reached a cell concentration of 10^8/ml. The cells were centrifuged, washed twice with minimal medium in the absence of glucose and amino acids and, finally, resuspended in one-tenth the original volume of the last medium.

Aliquots of 0.1 ml from each segregated culture were plated on petri dishes containing minimal medium supplemented either with (a) 20 amino acids without methionine, (b) 20 amino acids without threonine, (c) 20 amino acids without methionine and threonine, and (d) 20 amino acids as a control of the number of cells per plate. From each of the plates (a, b, c) one colony able to grow in the absence of one of the requirements was taken. Each colony was resuspended in 1 ml of minimal medium suplemented with glucose and amino acids except the one(s) for which they had become prototrophic and the requirements were again tested in solid medium. A colony was taken, grown in liquid medium till exponential phase and an aliquot was stored in 50% glycerol at −20 °C.

Petri dishes were seeded with the different bacterial derivatives and assayed by spot test for their capacity to allow the growth of representative *sus* mutants of phage Φ29 from the collection of Moreno *et al.* [13], using as a control of negative and positive growth, respectively, plates seeded with *B. subtilis* 110NA *su*$^-$ and *B. subtilis* MO-99 *su*$^{+3}$. The bacteria which did not allow the growth of some of the *sus* mutants were selected and again checked by direct plating of the *sus* mutants. In this way, strain *B. subtilis* 168 MO-101-P *spoA*$^-$ *thr*$^-$ [*met*$^-$]$^+$ *su*$^{+44}$, which did not allow the growth of mutant *susB*47, was selected.

Complementation Tests

Qualitative Assay. B. subtilis 110NA *su*$^-$ (about 10^8 bacteria), preinfected with a *sus* mutant ($2-5\times10^7$ pfu/plate) were plated on petri dishes. Individual plaques of the mutants to be tested by complementation, previously plated on the permissive strain, were transferred with a sterile tooth-pick to the plate containing the preinfected non-permissive bacteria and, as a control, to two other plates containing uninfected *B. subtilis su*$^-$ and *su*$^+$, respectively.

Quantitative Assay. This was carried out as previously described [13].

Recombination

Two-factor crosses were carried out as described [13].

Ultraviolet Irradiation and Labelling of Bacteria

The bacteria, *B. subtilis* MO-101-P *su*$^-$, MO-101-P *su*$^{+44}$ or MO-99 *su*$^{+3}$, were grown in minimal medium and irradiated prior to infection with ultraviolet light for the time indicated in each case, as described [15]. The irradiated bacteria were resuspended, at 5×10^8 cells/ml, in minimal medium containing 0.5 mM amino acids, except leucine which was 0.01 mM, infected with phage Φ29 or with different *sus* mutants at a multiplicity of 20 and shaken at 37 °C. A control was kept uninfected. At 15 min, samples were removed and the cultures infected with the *sus* mutants were labelled with [^{14}C]leucine (7.5 μCi/ml; 0.02 mM) and those infected with wild-type phage or the uninfected cultures were labelled with [^{3}H]leucine (25 μCi/ml; 0.02 mM). At 60 min (except where indicated otherwise) the bacteria were cooled in a bath of ice-water and sedimented by centrifugation. Each pellet was resuspended in half the original volume of a buffer containing 0.01 M sodium phosphate, pH 7.2, 1 mM EDTA, 0.58 mM phenylmethylsulfonyl fluoride and lysozyme, 500 μg/ml, incubated for 2 h at 0 °C, frozen and thawed three times, treated with pancreatic RNase (10 μg/ml) for 30 min at 0 °C and precipitated with 10% trichloroacetic acid. The precipitate was washed once with ether/ethanol (1/1) and twice with

ether/ethanol (3/1). The final precipitate was dried under a nitrogen stream and prepared for gel electrophoresis as indicated below.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out in a discontinuous pH system on 10-cm-long gels containing 10% acrylamide, 0.25% *N,N'*-methylenebisacrylamide and 0.1% sodium dodecylsulfate [18].

The samples for electrophoresis were prepared by heating for 2 min in a bath of boiling water in a buffer containing 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) sodium dodecylsulfate, 5% (v/v) 2-mercaptoethanol and 6 M urea. Electrophoresis was carried out at a constant voltage of 90 *V* for approximately 5.5 h, until the tracking dye ran out of the gel.

In some cases, gel electrophoresis in slabs 30-cm long and 1.5-mm thick was carried out (Carrascosa, J. L., Camacho, A., Moreno, F., Jiménez, F., Mellado, R. P., Viñuela, E. & Salas, M., unpublished). The separation gel was prepared by forming a linear gradient with two solutions, one containing 20% acrylamide and 0.33% *N,N'*-methylenebisacrylamide and the other containing 10% acrylamide and 0.25% *N,N'*-methylenebisacrylamide in the presence of 0.325 M Tris-HCl, pH 8.8, 0.1% sodium dodecylsulfate, 0.05% tetramethylethylenediamine and 0.0125% ammonium persulfate. The stacking gel was prepared as described [18]. The electrophoresis buffer contained 0.025 M Tris/0.192 M glycine, pH 8.3. The samples for electrophoresis were prepared as indicated above. 0.075–0.1 ml was loaded on each 4-mm-wide sample well. Electrophoresis was carried out at room temperature at a constant current of 20 mA per slab for 15 h. After electrophoresis, the gel slabs were dried under vacuum and autoradiographs were obtained on Kodirex X-ray film [19]. Densitometry was carried out with a Chromoscan MKII densitometer at 610–690 nm.

RESULTS

Isolation of B. subtilis su^{+44}

Among 148 revertants selected after mutagenesis of *B. subtilis* 168 MO-101-P *spoA*$^-$ *met*$^-$ *thr*$^-$ *su*$^-$, 91 were able to grow in the absence of threonine (from which 84 were *su*$^-$ and 7 were *su*$^+$), 26 were able to grow in the absence of methionine (from which 20 were *su*$^-$ and 6 were *su*$^+$) and 31 were able to grow in the absence of both amino acids (from which 20 were *su*$^-$ and 11 were *su*$^+$). Among the 24 *su*$^+$ revertants, only one, *B. subtilis* 168 MO-101-P *spoA*$^-$ *thr*$^-$ [*met*$^-$]$^+$ *su*$^{+44}$, was unable to allow the growth of one of the *sus* mutants (*susB*47) isolated on *su*$^{+3}$ by Moreno *et al.* [13]. All the other mutants tested from this collection grow on both *su*$^{+3}$ and *su*$^{+44}$, sometimes with slightly different efficiencies (Table 1). These different plating efficiencies on the two strains depend on the mutant and not on the cistron, suggesting that the amino acid inserted as a result of the suppression is more or less functional depending on the location of the mutation.

Table 1. *Efficiency of plating on* B. subtilis su^{+44} *of* sus *mutants isolated on* B. subtilis su^{+3}
The efficiency of plating was calculated for each mutant relative to 1. The order of the cistrons, from top to bottom, correspond to that obtained by two-factor and three-factor crosses [13]

Mutant	Efficiency of plating	
	su^{+44}	*su*$^{+3}$
sus$^+$	1.0	1.0
*F*513	0.9	1.0
*K*91	1.0	1.0
*K*442	0.6	1.0
*O*56	1.0	1.0
*A*422	1.0	1.0
*E*136	1.0	1.0
*E*74	1.0	1.0
*E*138	1.0	0.2
*H*542	1.0	1.0
*H*525	0.8	1.0
*B*47	10^{-5}	1.0
*L*53	1.0	0.5
*L*600	1.0	1.0
*M*1241	1.0	1.0
*N*212	0.9	1.0
*N*345	1.0	1.0
*D*241	1.0	1.0
*D*545	0.6	1.0
*P*112	1.0	1.0

Proteins Induced after Infection of B. subtilis su$^-$ *and* su^{+44} *with Φ29 Mutants* susN*212 and* susB*47*

The lack of growth of mutant *susB*47 on *B. subtilis su*$^{+44}$ could be due to the fact that either this suppressor was unable to recognize the nonsense codon present in this mutant or that, having recognized it, an amino acid were inserted which made an inactive protein. To decide between these two possibilities, the proteins synthesized in ultraviolet-irradiated bacteria infected with mutant *susB*47 were analyzed by gel electrophoresis. Bacteria infected with mutant *susN*212, which grows on both suppressor strains, were used as a positive control.

Fig. 1 A shows that after infection of irradiated *B. subtilis su*$^-$ with either mutant *susN*212 or *susB*47 no phage development took place. Fig. 1 B shows the results obtained after infection of *B. subtilis su*$^{+44}$ with these two mutants; in this case, phage development takes place after infection with mutant *susN*212 but not with *susB*47.

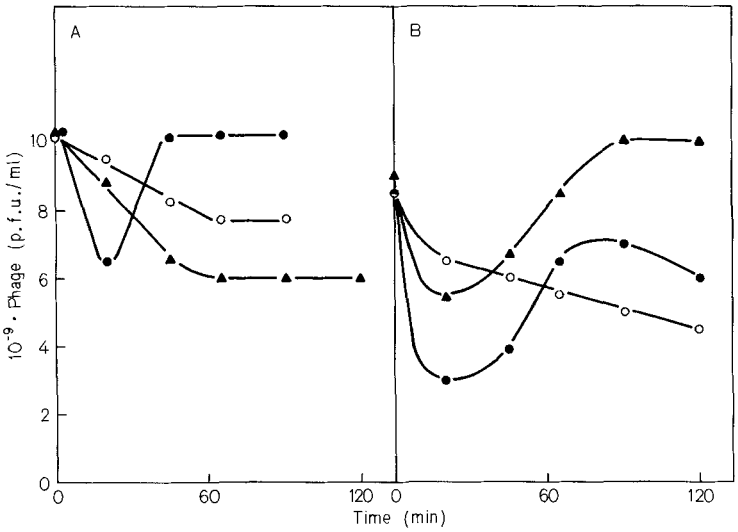

Fig. 1. *Phage development in ultraviolet-irradiated* B. subtilis su⁻ *or* su^{+44} *infected by wild-type phage or by mutants* susN*212 or* susB*47*. (A). *B. subtilis* MO-101-P *su*⁻ was irradiated with ultraviolet light for 10.5 min and infected with wild-type phage (●) or with mutants *susN*212 (▲) or *susB*47 (○). At the indicated times, aliquots were taken to determine total phage after lysis with lysozyme [14] (B). *B. subtilis* MO-101-P *su*$^{+44}$ was irradiated for 9 min and infected as in (A).

Fig. 2 (A–C) shows the proteins synthesized after infection of *B. subtilis* MO-101-P *su*⁻ with mutant *susN*212. As was shown previously in the case of strain 110NA *su*⁻ [10], no protein II, now renamed pN, is synthesized. On the contrary, when mutant *susN*212 infects *B. subtilis* MO-101-P *su*$^{+44}$ a considerable amount of protein pN is synthesized (Fig. 2, D–F). From the ratio of protein HP3 to protein pN in wild-type infected cells and that of these proteins in *susN*212-infected cells, a suppression of 64% can be estimated, a value about 8 times higher than that obtained in the case of the *B. subtilis* strain *su*$^{+3}$ [10].

With such a high suppression efficiency one should be able to decide between the two possibilities raised above for the lack of suppression of mutant *susB*47 in strain *su*$^{+44}$. Infection of *B. subtilis* MO-101-P *su*⁻ with mutant *susB*47 (Fig. 3A–C) does not give place to the synthesis of protein NP1 and, instead, it produces a lower-molecular-weight fragment (NP1*) as was previously shown in the case of *B. subtilis* 110NA *su*⁻ [10]. No synthesis of protein NP1 takes place either after infection of *B. subtilis su*$^{+44}$ with mutant *susB*47 (Fig. 3, D–F), indicating that the absence of development of the mutant in this suppressor strain is due to the fact that the suppressor is unable to recognize the nonsense codon present in mutant *susB*47 rather than to the insertion of a nonfunctional amino acid. A suppression of about 12% had been obtained for mutant *susB*47 in strain *B. subtilis su*$^{+3}$ [10].

Revertants of Mutant susB*47*

The above results are consistent with the possibility that mutant *susB*47 contains an ochre mutation; strain *su*$^{+3}$ being an ochre suppressor of low efficiency of suppression (about 10%) and strain *su*$^{+44}$ an amber suppressor with a high efficiency of suppression (about 60%), *su*$^{+44}$ is unable to suppress the ochre mutation present in mutant *susB*47. If this were the case, one should be able to obtain from mutant *susB*47 an amber derivative which would grow on both *su*$^{+44}$ and *su*$^{+3}$ strains, after the occurrence of an A→G transition at the nonsense codon.

Out of 500 revertants from mutant *susB*47, able to grow on *B. subtilis su*$^{+44}$, 26 were still *sus* mutants, unable to grow on *B. subtilis su*⁻ but able to develop on *su*$^{+44}$ as well as on *su*$^{+3}$. By spot test complementation these mutants were shown to belong to cistron B and one of them, *susB*610, was studied further. Since mutants *susB*610 and *susB*47 do not recombine, both mutations are probably at the same location.

Proteins Synthesized after Infection of B. subtilis su⁻, su^{+44} *and* su^{+3} *with Mutant* susB*610*

Fig. 4 (A–C) shows the phage development after infection of *B. subtilis su*⁻, *su*$^{+44}$ and *su*$^{+3}$, respectively, with mutant *susB*610. Contrary to what happens with mutant *susB*47 (Fig. 1B), mutant *susB*610 can grow on *B. subtilis su*$^{+44}$.

The proteins synthesized after infection of *B. subtilis su*⁻ with mutant *susB*610 are shown in Fig. 5 (A–C). As can be seen, no protein NP1 is synthesized; instead, the 75000-molecular-weight fragment (NP1*) [10] appears.

When mutant *susB*610 infects *B. subtilis su*$^{+3}$ a small amount of protein NP1 appears (Fig. 6, D–F). From the ratio of the radioactivity present in protein

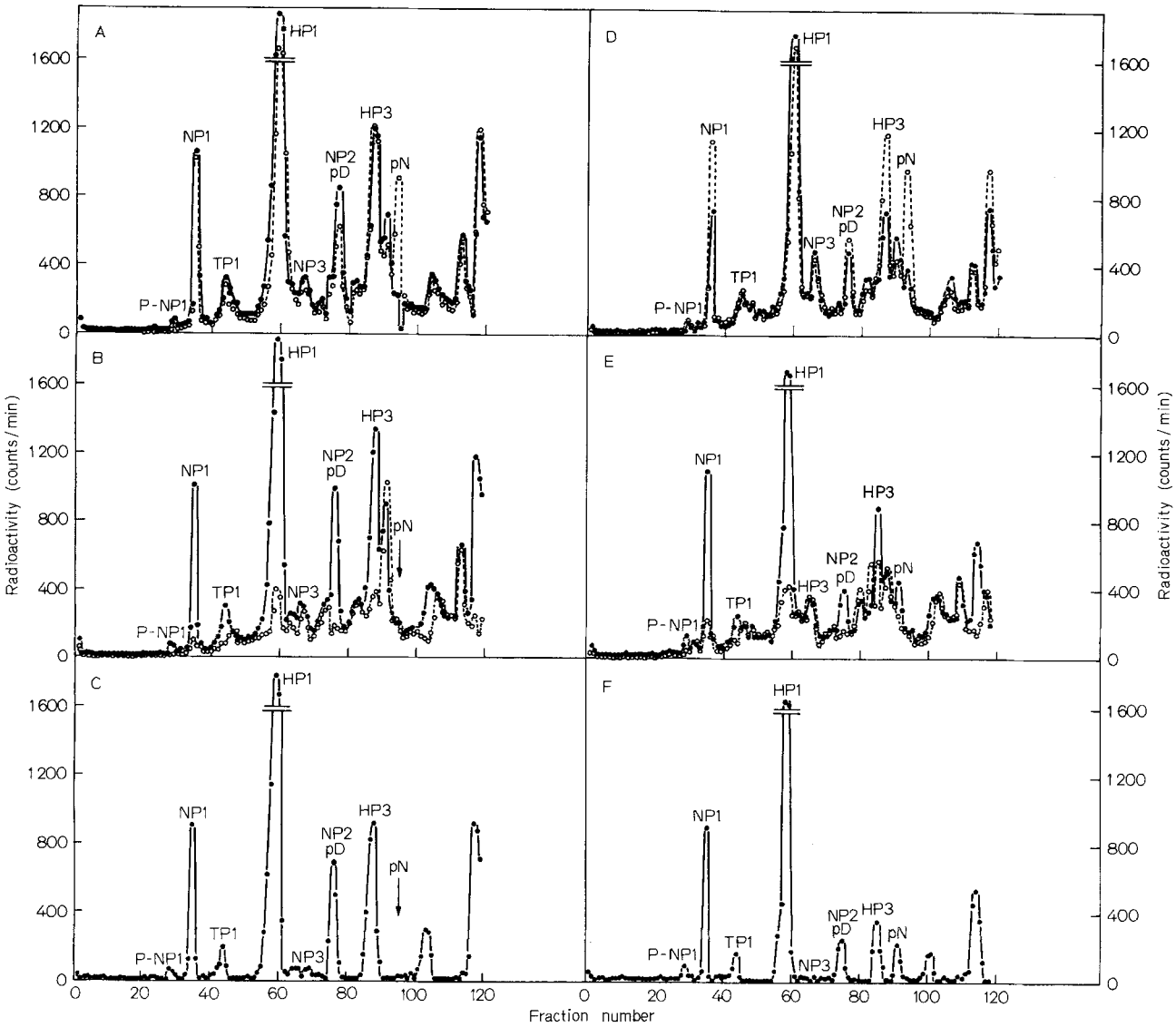

Fig. 2. *Gel electrophoresis of the proteins induced by infection of ultraviolet-irradiated* B. subtilis su⁻ *or* su^{+44} *with mutant* susN*212. B. subtilis* MO-101-P *su*⁻ or *su*$^{+44}$ cells, irradiated for 10.5 and 9 min, respectively, were infected with mutant *susN*212 and labelled with [^{14}C]leucine from 15 to 60 min after infection. Unifected cells or cells infected with *Φ* 29 wild type were labelled with [^{3}H] leucine at the same time. Tube electrophoresis was carried out as indicated in Materials and Methods. (A), Coelectrophoresis of proteins from *susN*212-infected *B. subtilis su*⁻ (●——●) and wild-type infected *su*⁻ (O– – –O). (B) *susN*212-infected *su*⁻ (●——●) and uninfected *su*⁻ (O– – –O). (C) *susN*212-specific radioactivity in *su*⁻ bacteria calculated according to the method of Mayol and Sinsheimer [21]. (D) *susN*212-infected *B. subtilis su*$^{+44}$ (●——●) and wild-type infected *su*$^{+44}$ (O– – –O). (E) *susN*212-infected *su*$^{+44}$ (●——●) and uninfected *su*$^{+44}$ (O– – –O). (F) *susN*212-specific radioactivity in *su*$^{+44}$ bacteria calculated as described [21]

NP1 to that present in protein NP1 and the 75000-molecular-weight fragment (NP1*) (Fig. 6F), a suppression of 8% can be estimated, a value close to that obtained for the suppression of mutant *susB*47 [10].

Fig. 6(A–C) shows the proteins induced after infection of *B. subtilis su*$^{+44}$ with mutant *susB*610. In this case, a considerable amount of protein NP1 is synthesized. By calculating the radioactivity under the NP1 peak and the NP1 fragment (Fig. 6C) a suppression of 57% is obtained, about 7 times larger than that obtained with strain *su*$^{+3}$. This result agrees very well with the suppression value obtained for mutant *susN*212.

Suppression of Mutants Isolated on B. subtilis su^{+44} *by* B. subtilis su^{+3}

The above results strongly suggest that the *B. subtilis* strain *su*$^{+3}$ contains an ochre suppressor and *B. subtilis su*$^{+44}$ an amber suppressor. Mutant *susB*47 would contain an ochre mutation being suppressed by *su*$^{+3}$ but not by *su*$^{+44}$. In the case of revertant *susB*610, an A to G transition in the third base of the triplet would give place to an amber mutation which would be now suppressed by both suppressor strains, *su*$^{+3}$ (ochre) and *su*$^{+44}$ (amber).

By using the strong suppressor *su*$^{+44}$ we have isolated a new collection of *Φ*29 nonsense mutants.

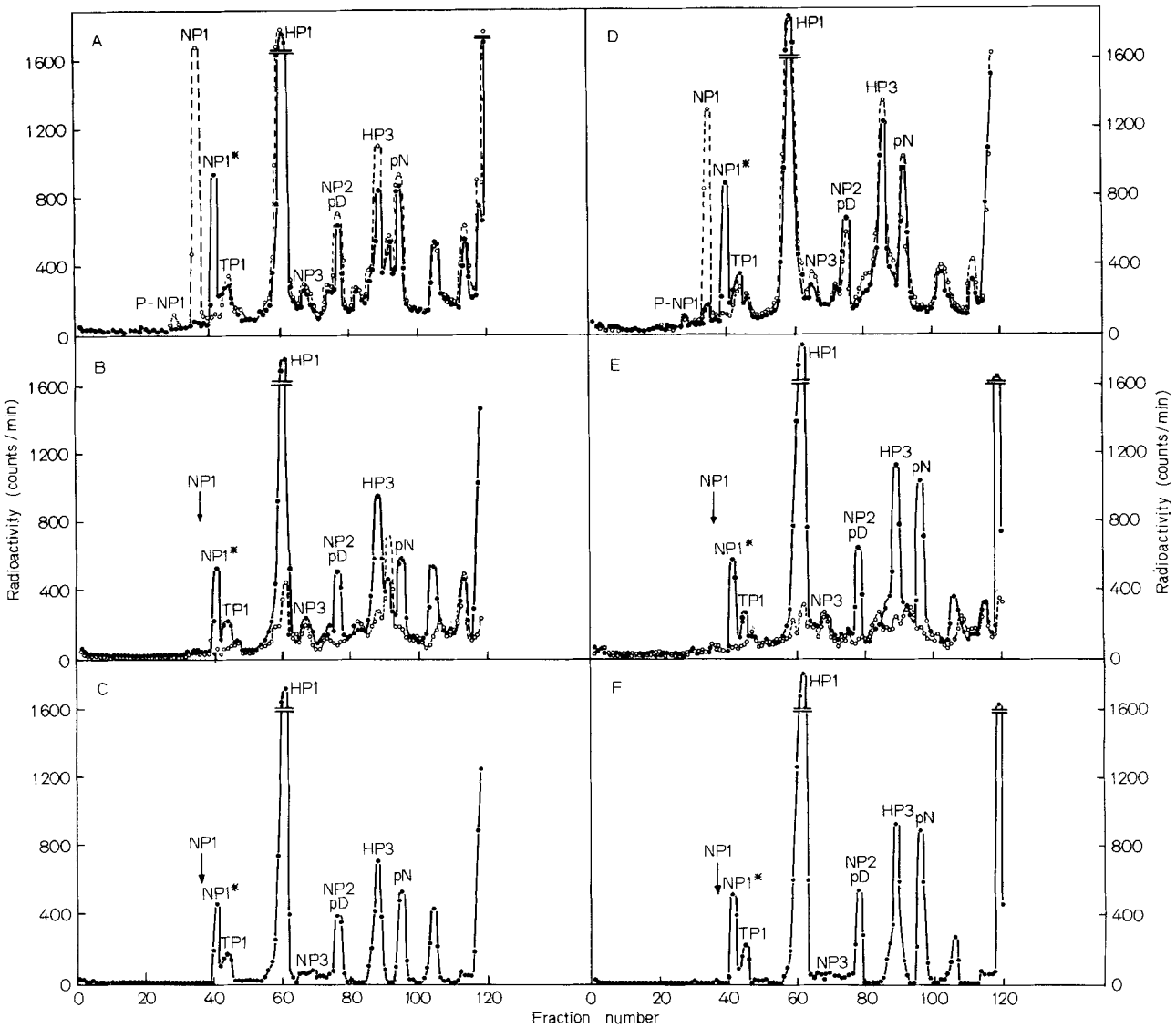

Fig. 3. *Gel electrophoresis of the proteins induced by infection of ultraviolet-irradiated* B. subtilis su⁻ *or* su^{+44} *with mutant* susB*47*. Cells were irradiated, infected with mutant *susB*47 or with wild-type phage and labelled as desribed in Fig. 2. Tube electrophoresis was carried out as indicated in Materials and Methods. (A). Coelectrophoresis of *susB*47-infected *B. subtilis su*⁻ (●——●) and wild-type infected *su*⁻ (O– – –O). (B). *susB*47-infected *su*⁻ (●——●) and uninfected *su*⁻ (O– – –O). (C). *susB*47-specific radioactivity in *su*⁻ bacteria calculated as described [21]. (D). *susB*47-infected *B. subtilis su*$^{+44}$ (●——●) and wild-type infected *su*$^{+44}$ (O– – –O). (E). *susB*47-infected *su*$^{+44}$ (●——●) and uninfected *su*$^{+44}$ (O– – –O). (F). *susB*47-specific radioactivity in *su*$^{+44}$ bacteria calculated as described [21]

Table 2 gives a summary of the results obtained until now, with the pattern of suppression on strain *su*$^{+3}$. As can be seen, not all the mutants isolated on *B. subtilis su*$^{+44}$, which would be amber mutants, are suppressed on *B. subtilis su*$^{+3}$. This is contrary to what one would expect for an ochre suppressor, unless either the amino acid inserted by suppressor *su*$^{+3}$ made the protein nonfunctional or the proteins suppressed in strain *su*$^{+3}$ were not made in the amount required for phage development. The last possibility is unlikely since in all cistrons there is at least one mutant which is suppressed by strain *su*$^{+3}$. In any case, one should obtain suppression of the mutation at the level of the protein. To test this possibility, the proteins synthesized after infection of *B. subtilis su*⁻, *su*$^{+44}$ and *su*$^{+3}$ with mutant *susG*22, which is suppressed by strain *su*$^{+44}$ but not by strain *su*$^{+3}$ (Table 2 and Fig. 4), were analyzed by polyacrylamide gel electrophoresis. Fig. 5 (D–F) shows that proteins HP1 and HP3 are not synthesized after infection of *B. subtilis su*⁻ with mutant *susG*22. The possibility that the absence of both proteins is due to a double mutation is unlikely since the same two proteins are also lacking after infection of *B. subtilis su*⁻ with other *sus* mutants in cistron G [20] (and Carrascosa *et al.*, unpublished). Two other possibilities, among others, are open: (a) a polar effect of the *sus* mutation in cistron G coding for one of the two head proteins, towards a new

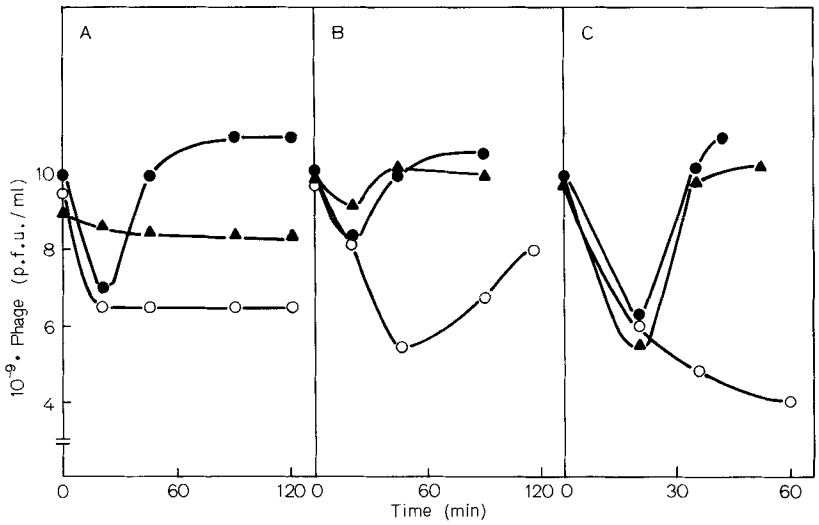

Fig. 4. *Phage development in ultraviolet-irradiated* B. subtilis su$^-$, su^{+44} *and* su^{+3} *infected by mutants* susB*610 or* susG*22*. (A) *B. subtilis* MO-101-P *su*$^-$ was irradiated with ultraviolet light for 10.5 min and infected with wild-type phage (●) or with mutants *susB*610 (▲) or *susG*22 (○). At the indicated times, aliquots were taken to determine total phage after lysis with lysozyme [14]. (B) *B. subtilis* MO-101-P *su*$^{+44}$ was irradiated for 10.5 min and infected as in (A). (C) *B. subtilis* MO-99 *su*$^{+3}$ was irradiated for 9 min and infected as in (A)

Table 2. *Supression by* B. subtilis su^{+3} *of Φ 29 mutants isolated on* B. subtilis su^{+44}

The values refer to the number of mutants isolated on *B. subtilis su*$^{+44}$ which are suppressed and not suppressed, respectively, on *B. subtilis su*$^{+3}$. The order of the cistrons from top to bottom, corresponds to that obtained by two-factor and three-factor crosses [13]

Cistron	*su*$^{+3}$	
	suppressed	not suppressed
F	3	0
K	3	2
O	2	0
Q	2	0
J	1	0
G	4	2
A	1	2
E	3	2
H	3	0
B	2	0
I	7	2
L	3	1
N	8	0
D	3	3

cistron as yet unidentified, coding for the other protein; (b) cistron G could code for the two head proteins, HP1 and HP3, giving place to a precursor which would be further cleaved yielding proteins HP1 and HP3. Fig. 7 (A – C) shows the synthesis of proteins HP1 and HP3 after infection of *B. subtilis su*$^{+44}$ with mutant *susG*22. From the ratio of proteins HP1 and HP3, respectively, to protein pN in wild-type infected cells and that of those proteins in *susG*22-infected cells, a suppression of about 48% and 37%, respectively, for proteins HP1 and HP3, can be estimated. These values are similar to those obtained for mutants *susN*212 and *susB*610 on *B. subtilis su*$^{+44}$ (64 and 57%, respectively).

Fig. 7 (D – F) shows the proteins synthesized after infection of *B. subtilis su*$^{+3}$ with mutant *susG*22. As can be seen, a small level of suppression in the synthesis of proteins HP1 and HP3 is obtained. The level of suppression is calculated to be about 10% for both proteins HP1 and HP3, a value very similar to that obtained for mutants *susB*47 and *susN*212 [10] and for mutant *susB*610 reported here.

The above results strongly suggest that the failure for mutant *susG*22 to grow on strain *su*$^{+3}$ is not due to inability of such a strain to suppress the *sus* mutation, but rather to the insertion of a non-functional amino acid. In accordance with this, none of the 500 revertants isolated from mutant *susG*22 able to grow on *B. subtilis su*$^{+3}$ were *sus* mutants but rather wild-type revertants.

To test whether or not the fact that other mutants isolated on *B. subtilis su*$^{+44}$ did not plate on *B. subtilis su*$^{+3}$ (Table 2) was due to the same reason shown for mutant *susG*22, two other mutants in cistron D were analyzed. The protein product of this cistron, pD, is not resolved from NP2 by electrophoresis in a tube [15], but these two proteins are resolved by slab gel electrophoresis (Carrascosa *et al.*, unpublished). The proteins synthesized after infection of *B. subtilis su*$^{+3}$ with mutants *susD*121 and *susD*172, which did not plate on *B. subtilis su*$^{+3}$ and, as a control, with wild-type phage, were labelled with [^{14}C]leucine and analyzed by slab gel electrophoresis. Fig. 8 shows the densitometry of the autoradiograph of the gel slabs.

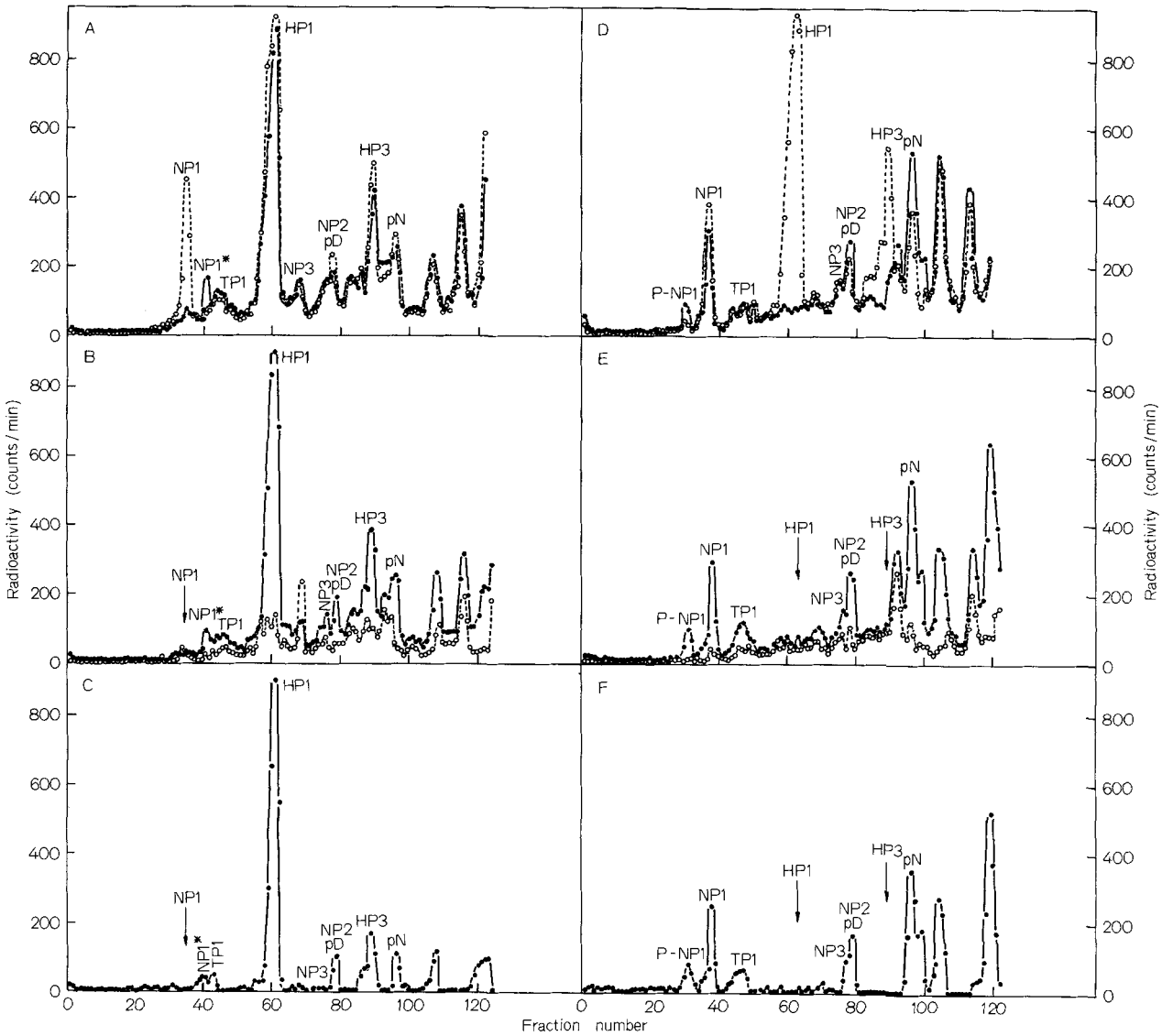

Fig. 5. *Gel electrophoresis of the proteins induced by infection of ultraviolet-irradiated* B. subtilis su⁻ *with mutants* susB*610 and* susG*22. B. subtilis* MO-101-P *su*⁻ was irradiated and infected with mutants *susB*610, *susG*22 or with wild-type phage. Cells infected with mutant *susB*610 or with wild-type phage were labelled from 15 to 60 min. Cells infected with mutant *susG*22 were labelled from 35 to 80 min. The uninfected cells were labelled during both time periods. Electrophoresis in tube was carried out as described in Materials and Methods. (A) Coelectrophoresis of proteins from *susB*610-infected *B. subtilis su*⁻ (●——●) and wild-type infected *su*⁻ (O- - -O). (B) *susB*610-infected *su*⁻ (●——●) and uninfected *su*⁻ (O- - -O). (C) *susB*610 specific radioactivity calculated as described [21]. (D) *susG*22-infected *su*⁻ (●——●) and wild-type infected *su*⁻ (O- - -O). (E) *susG*22-infected *su*⁻ (●——●) and uninfected *su*⁻ (O- - -O). (F) *susG*22-specific radioactivity calculated as described [21]

Infection with mutant *susD*121 (Fig. 8B) produces the synthesis of a protein peak at the position of protein pD, present in wild-type-infected cells (see Fig. 8A); this peak is not produced in uninfected cells (Fig. 8C). It can also be seen in Fig. 8B that there exists a polypeptide of smaller molecular weight than pD, not present either in wild-type-infected cells or in uninfected cells; this peak could be a fragment of protein pD (pD*). Either from the ratio of protein pD to NP2 in wild-type-infected cells and that in *susD*121-infected cells or from the ratio of protein pD to fragment (pD*) and protein pD in mutant-infected cells, one can get a rough estimate of the suppression as being approximately 20%. The determination of the suppression in this case is much less accurate than when double-label experiments with infected and uninfected cells are carried out, since the contribution of the proteins from uninfected bacteria is difficult to quantify. Infection with mutant *susD*172 gave similar results, except that no fragment of protein pD was seen. The calculated efficiency of suppression in this case was approximately 10%.

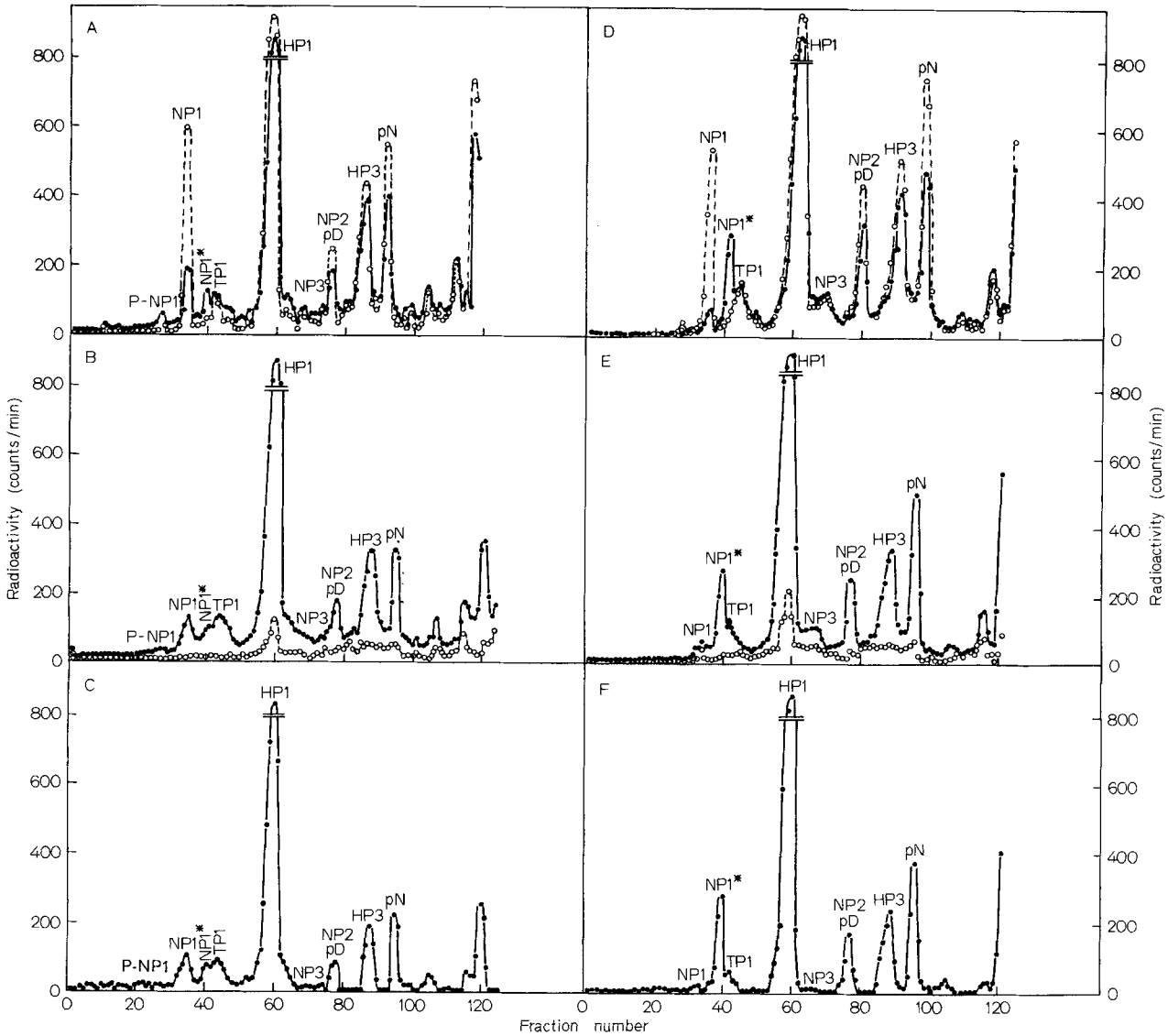

Fig. 6. *Gel electrophoresis of the proteins induced by infection of ultraviolet-irradiated* B. subtilis su^{+44} *or* su^{+3} *with mutant* susB*610*. *B. subtilis* MO-101-P *su*$^{+44}$ or MO-99 *su* $^{+3}$ were irradiated for 10.5 and 9 min, respectively, infected with mutant *susB*610 or with wild-type phage and labelled from 15 to 60 min postinfection in the case of *B. subtilis su*$^{+44}$ and from 15 to 25 min in the case of *B. subtilis su*$^{+3}$. Uninfected cells were labelled at the same times as the infected cells, depending on the bacteria. Electrophoresis in tube was carried out as described in Materials and Methods. (A) Coelectrophoresis of proteins from *susB*610-infected *B. subtilis su*$^{+44}$ (●——●) and wild-type infected *su*$^{+44}$ (○– – –○). (B) *susB*610-infected *su*$^{+44}$ (●——●) and uninfected *su*$^{+44}$ (○– – –○). (C) *susB*610-specific radioactivity in *su*$^{+44}$ bacteria calculated as described [21]. (D) *susB*610-infected *su*$^{+3}$ (●——●) and wild-type infected *su*$^{+3}$ (○– – –○). (E) *susB*610-infected *su*$^{+3}$ (●——●) and uninfected *su*$^{+3}$ (○– – –○). (F) *susB*610-specific radioactivity in *su*$^{+3}$ bacteria calculated as described [21]

DISCUSSION

Two nonsense suppressors have been characterized in *B. subtilis:* strain *su*$^{+3}$ from Georgopoulos [8] with an efficiency of suppression of about 10% [10], and strain HA 101 B from Okubo and Yanagida [7] with an efficiency of 27% [11]. The new suppressor strain reported in this paper, *B. subtilis su*$^{+44}$, has an efficiency of suppression of about 50%, higher than that obtained with the two other nonsense suppressors. This suppressor strain has been isolated by treatment of the *B. subtilis* strain MO-101-P *su*$^{-}$ with ethyl methanesulfonate, a mutagen shown by Osborn *et al.* [17] to produce in *E. coli* 90% of amber suppressors.

Comparing with the probability of chain propagation in different *E. coli* suppressors [1–4], the high efficiency of suppression obtained with *B. subtilis su*$^{+44}$ could suggest that we had isolated a suppressor of either UAG or UGA mutations. However, the fact that we have selected on strain *su*$^{+3}$ *Φ*29 nonsense mutants which are suppressed by strain *su*$^{+44}$, and one mutant, *susB*47, which is not suppressed by this strain,

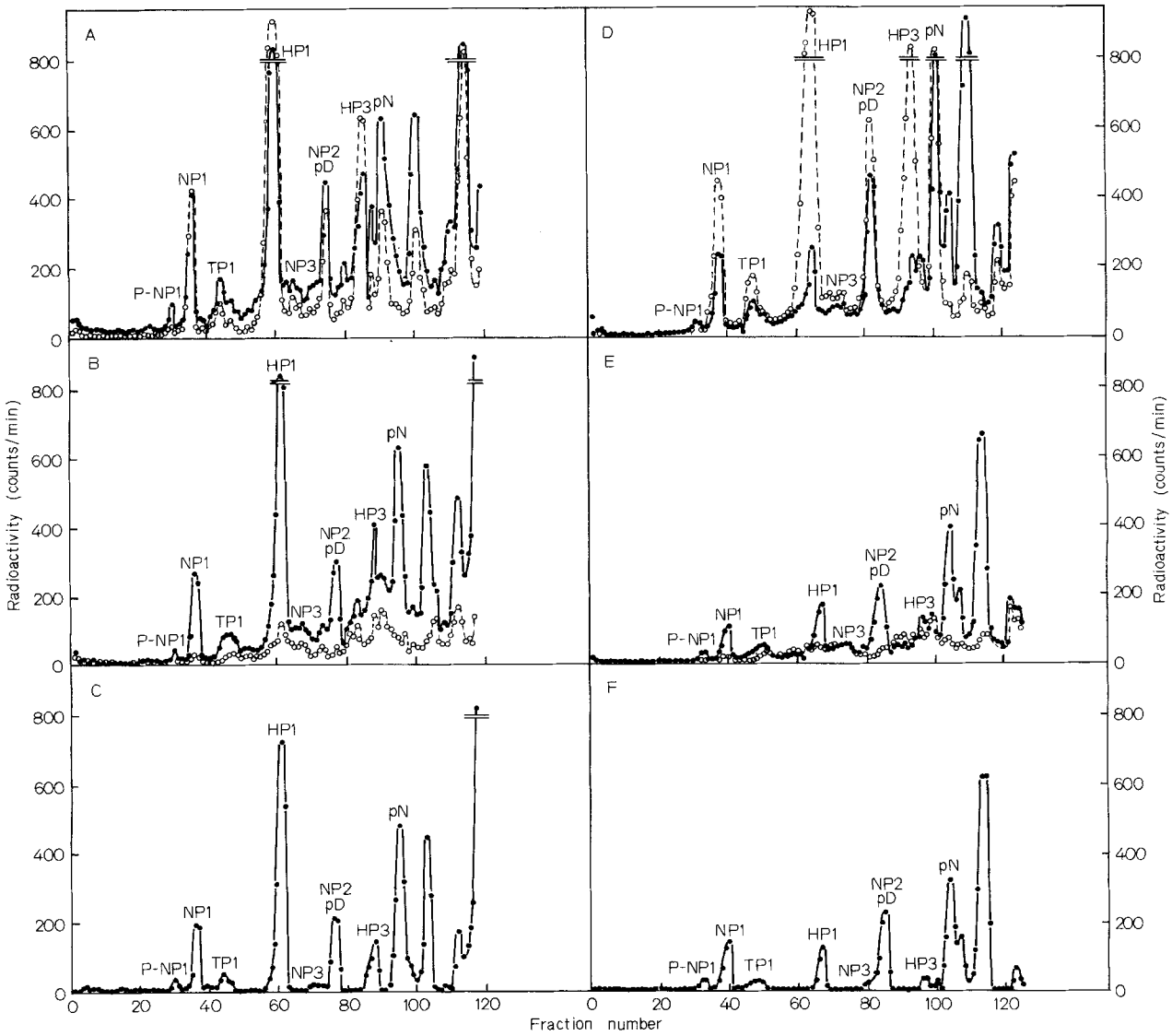

Fig. 7. *Gel electrophoresis of the proteins induced by infection of ultraviolet-irradiated B. subtilis* su^{+44} *or* su^{+3} with mutant susG22. *B. subtilis* MO-101-P *su*$^{+44}$ or MO-99-*su*$^{+3}$ were irradiated as described in Fig. 6 and infected with mutant *susG* 22 or with wild-type phage. Cells infected with mutant *susG* 22 were labelled from 35 to 80 min for *su*$^{+44}$ and from 15 to 40 min for *su*$^{+3}$. Cells infected with wild-type phage were labelled from 15 to 60 min for *su*$^{+44}$ and from 15 to 25 min for *su*$^{+3}$. Uninfected cells were labelled at the same times as the *susG* 22-infected cells. Electrophoresis in tube was carried out as described in Materials and Methods. (A) Coelectrophoresis of proteins from *susG* 22-infected *B. subtilis su*$^{+44}$ (●——●) and wild-type infected *su*$^{+44}$ (O---O). (B) *susG* 22-infected *su*$^{+44}$ (●——●) and uninfected *su*$^{+44}$ (O---O). (C) *susG* 22-specific radioactivity in *su*$^{+44}$ bacteria calculated as described [21]. (D) *susG* 22-infected *su*$^{+3}$ (●——●) and wild-type infected *su*$^{+3}$ (O---O). (E) *susG* 22-infected *su*$^{+3}$ (●——●) and uninfected *su*$^{+3}$ (O---O). (F) *susG* 22-specific radioactivity in *su*$^{+3}$ bacteria calculated as described [21]

discards the possibility of strain *su*$^{+44}$ having an UGA suppressor [3] and suggests that it may contain an UAG suppressor. In that case, mutant *susB*47 would contain an ochre mutation and strain *su*$^{+3}$ would be an ochre suppressor, a fact consistent with its low efficiency of suppression. If that were the case, one should be able to get nonsense revertants from mutant *susB*47 which would be suppressed by both strains *su*$^{+44}$ and *su*$^{+3}$. In fact, a collection of 26 of such revertants were found which plated on both *su*$^{+44}$ and *su*$^{+3}$ strains and by complementation were shown to belong to cistron B. On of such revertants, *susB*610, was analyzed further and shown not to recombine with mutant *susB*47, suggesting that mutations *susB*47 and *susB*610 have the same location. Moreover, the efficiencies of suppression of mutant *susB*610 by strains *su*$^{+44}$ and *su*$^{+3}$ were 57% and 8%, respectively, a pattern similar to that obtained with mutant *susN*212, suppressed by the two suppressor strains, and contrary to what happens with mutant *susB*47, which is not suppressed at all by strain *su*$^{+44}$, being suppressed about 12% by *su*$^{+3}$ [10]. All these results strongly suggest that strain *su*$^{+3}$ contains an ochre suppressor and strain *su*$^{+44}$ contains an amber suppressor. Mutant *susB*47 would have an UAA mutation which would revert to mutant *susB*610, with an amber mutation,

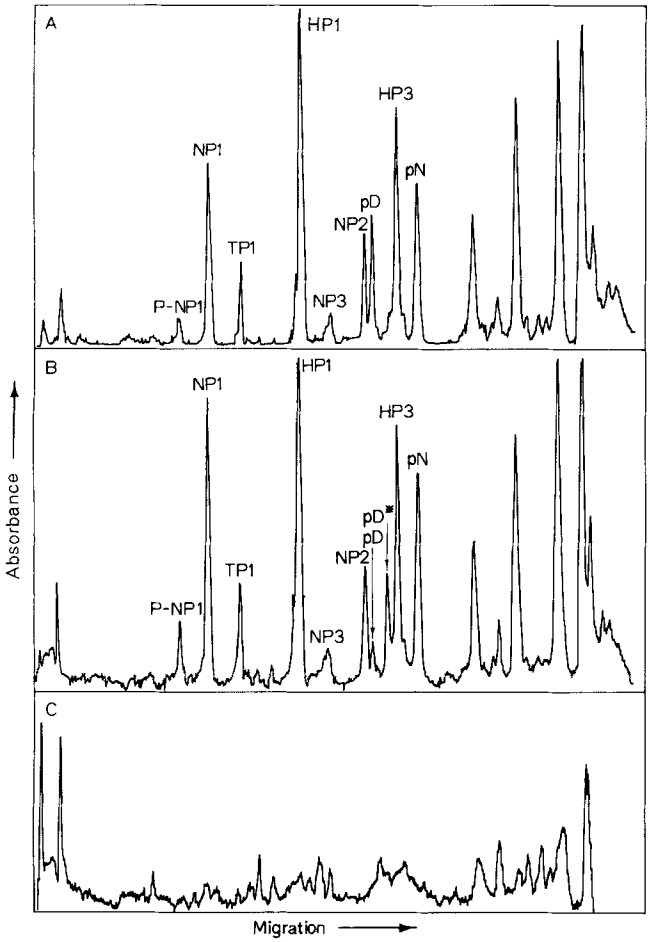

Fig. 8. *Densitometry of the proteins induced in ultraviolet-irradiated* B. subtilis su^{+3} *by infection with mutant* susD*121*. *B. subtilis* MO-99-*su*$^{+3}$ was irradiated for 9 min and infected with mutant *susD*121 (B) or with wild-type phage (A). Cells were labelled from 15 to 35 min after infection. Uninfected cells were also labelled at the same time (C). Slab gel electrophoresis, autoradiography and densitometry were carried out as described in Materials and Methods

UAG, which now would be suppressed both by the ochre (*su*$^{+3}$) and amber (*su*$^{+44}$) suppressor strains.

This otherwise straightforward scheme faces, however, a peculiar fact: among a collection of 59 nonsense mutants isolated on strain *su*$^{+44}$, 14 mutants distributed in 7 out of 14 cistrons did not plate on strain *su*$^{+3}$. A possibility was that the amino acid inserted by strain *su*$^{+3}$ would make the different proteins nonfunctional. To test this possibility the proteins synthesized after infection of *B. subtilis su*$^{+3}$ by mutants *susG*22, *susD*121 and *susD*172 were analyzed. In all cases threre was a suppression of 10–20% indicating that the lack of plating of these mutants on strain *su*$^{+3}$ is not due to a failure to suppress the mutation but rather, probably, to the insertion of an amino acid which makes a nonfunctional protein.

All the results presented are consistent with the idea that strains *su*$^{+44}$ and *su*$^{+3}$ contain an amber and ochre suppressor, respectively. However, to determine the exact nature of these suppressors, protein synthesis experiments will be carried out *in vitro* by using either the DNA from *E. coli* phages with known amber and ochre mutations and the tRNAs from the *su*$^{-}$, *su*$^{+3}$ or *su*$^{+44}$ *B. subtilis* strains or the DNA from *Φ*29 *sus* mutants in an *E. coli* cell-free system containing *E. coli* tRNA from ochre or amber suppressor strains.

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