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Genetic and Biochemical Characterization of Kirromycin Resistance Mutations in *Bacillus subtilis*

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Spontaneous mutations causing resistance to the EF-Tu-specific antibiotic kirromycin have been isolated and mapped in *Bacillus subtilis*. Three-factor transductional and transformational crosses have placed the *kir* locus proximal to *ery-1* and distal to *strA* (*rpsL*) and several mutations affecting elongation factors EF-G and EF-Tu, in the order:

$$cysA\ strA \left[\begin{matrix} fus\text{-}1 \\ ts\text{-}6(\text{EF-G}) \end{matrix} \right] [ts\text{-}5(\text{EF-Tu})]\ kir\ ery\text{-}1\ spcA.$$

Purified EF-Tu from mutant strains is more resistant to kirromycin as measured by in vitro protein synthesis and also shows a more acidic isoelectric point than wild-type EF-Tu. This indicates that the *kir* locus is the genetic determinant (*tuf*) for EF-Tu and that there is a single active gene for this enzyme in *B. subtilis*.

There are many similarities in the chromosome organization of the genetic determinants for the translational apparatus in procaryotes, especially in the *strA* (*rpsL*) region of *Escherichia coli* and *Bacillus subtilis* (34), where the map order for genes coding for several ribosomal proteins and protein synthesis elongation factors is identical (11, 20). However, all known genes for ribosomal proteins and elongation factors are clustered in the *strA-spcA* region of *B. subtilis* (11, 32-34), although a group of mutations possibly affecting ribosomal proteins has been mapped elsewhere (38). There are several clusters in *E. coli* (29).

The question of the genetic determinants for EF-Tu is significant in this regard. Genes for EF-Tu map next to those governing EF-G and *strA* in both *E. coli* and *B. subtilis*. However, there is also an EF-Tu gene (*tufB*) mapping in the *rif* region of *E. coli* (12, 21, 39), although in some *E. coli* strains there seems to be only active *tuf* gene (12).

We have previously mapped several conditional mutations affecting in vitro activity of EF-G and EF-Tu (11). The presence of such point mutations indicated the existence of one active EF-Tu gene. However, in the absence of any further evidence on the nature of the lesion affecting EF-Tu, we could not rule out the possibility that these mutations were in a gene for a modifier protein and not in the structural gene for EF-Tu.

Kirromycin is an antibiotic which affects EF-Tu in vitro (40), and mutations to kirromycin resistance in *E. coli* affect EF-Tu activity in vitro and map where *tufA* and *tufB* are found (12, 39).

This communication deals with the genetic mapping and biochemical characterization of mutations to kirromycin resistance in *B. subtilis*. We have found that mutations for kirromycin resistance map in the same chromosomal region as other mutations affecting EF-G and EF-Tu and also affect EF-Tu in vitro.

MATERIALS AND METHODS

Strains. Strain IS3 (Table 1) was used to obtain spontaneous kirromycin-resistant mutants. Overnight cultures grown in VY (28) medium were spread (10^8 colony-forming units per plate) onto TBAB agar (10) containing 200 μg of kirromycin per ml (for strain IS111), whereas 400 μg of methylkirromycin per ml was used to isolate a nonsibling mutant (strain IS115). Other strains are described in Table 1.

Genetic methods. *B. subtilis* bacteriophage AR9 (5) was used to prepare generalized transducing lysates, and transduction was performed as described previously (10, 15). All media, transforming DNA, competent cells, and procedures for transformation were performed as previously described (10). Temperature-resistant recombinants were selected and scored at 53°C on TBAB plates as described previously (11). Expression times for the primary selection of antibiotic-resistant recombinants were 2.5 to 3 h, and the following antibiotics and concentrations (in micrograms per milliliter) were used for primary selection and for scoring as unselected markers: streptomycin, 1,000; spectinomycin, 100; erythromycin, 1.0; fusidic

TABLE 1. *Bacterial strains*

Strain	Parent	Description[a]	Source
IS1		*trpC2 thr-5*	9
IS2	IS1	*trpC2 thr-5 strA*	transformation for *strA*
IS3		*cysA14*	15
IS6		*trpC2 thr-5 cysA14*	11
IS13	IS6	*trpC2 cysA14 ts-5*	11
IS16	IS6	*trpC2 cysA14 ts-6*	11
IS26	IS2	*trpC2 thr-5 strA spcA*	transformation for *spcA*
IS48		*fus-1*	17
IS111	IS3	*cysA14 kir-2*	spontaneous mutation to 200 μg of kirromycin/ml
IS115	IS3	*cysA14 kir-7*	spontaneous mutation to 400 μg of methylkirromycin per ml
IS116	IS2	*trpC2 thr-5 strA kir-2*	transformation for *kir-2*
IS121		*ery-1*	15

[a] Gene symbols are as described in Demerec et al. (8). *kir* refers to kirromycin resistance.

acid, 2; kirromycin or methylkirromycin, 200.

Growth of cells for biochemical studies. Cells were grown in VY medium to late-log phase (approximately 10^9 cells per ml). They were harvested and washed with high- and low-salt buffers containing glycerol to minimize proteolytic activity, as previously described (31). Washed cells were frozen at −80°C before use.

Purification of EF-Tu. All procedures were performed at 4°C except the affinity column step, which was carried out at room temperature. A 6-g quantity of cells was suspended in 3 volumes of a solution containing 10 mM magnesium acetate, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol, and 10 mM tris-(hydroxymethyl)aminomethane (Tris), pH 7.4. The cells were lysed by passage through a French pressure cell at 20,000 lb/in.^{2} The lysate was collected into test tubes containing sufficient Macaloid (Baroid) to give a final concentration of 1 mg/ml. Ribonuclease-free deoxyribonuclease (Worthington Biochemicals Corp.) was added to give a final concentration of 5 μg/ml, and the lysate was centrifuged for 40 min at 20,000 rpm in a Sorvall SS34 rotor. The supernatant solution (S-30) was centrifuged for 3 h at 40,000 rpm in a Spinco Ti 50 rotor. The upper two-thirds of the supernatant solution was fractionated with solid ammonium sulfate, and the 35 to 65% saturated ammonium sulfate precipitate was collected. This precipitate was suspended in 12 ml of Sepharose dialysis buffer (10 mM $MgCl_2$, 0.35 M NaCl, 5 mM 2-mercaptoethanol, 50 mM Tris, pH 7.8; 18). The following steps were carried out at room temperature. The suspension was added to an equal volume of packed GDP-Sepharose prepared by the method of Jacobson and Rosenbusch (18).

GDP was oxidized with sodium periodate and was covalently linked to AH-Sepharose 4B (Pharmacia Fine Chemicals, Inc.) by sodium borohydride reduction. The slurry was placed in a dialysis tube and dialyzed against 1 liter of Sepharose dialysis buffer overnight with gentle rotation to insure complete mixing. The slurry was then packed into a column (2 by 5 cm), and the run-through fraction was collected. The column was then washed with 5 bed volumes of Sepharose dialysis buffer. Then 1 bed volume of the same buffer containing 100 μM GDP was run into the column and incubated for 1 h. This was followed by developing the column with 5 more bed volumes of dialysis buffer containing GDP.

The remaining steps were carried out at 4°C. Fractions containing EF-Tu activity were pooled and precipitated by adding ammonium sulfate to a 65% saturation, and the precipitate was suspended in TMS buffer (0.01 M magnesium acetate, 0.25 M sucrose, 5 mM 2-mercaptoethanol, 0.02 M Tris, pH 7.5) (4) and stored at −80°C.

Purification of EF-G. All steps were carried out at 4°C. Elongation factor EF-G was purified by pooling the run-through and the first washes of the GDP-Sepharose column before the addition of GDP (see above). The fraction precipitating at 65% saturation with ammonium sulfate was suspended in a solution containing 10 mM magnesium acetate, 0.15 M KCl, 5 mM 2-mercaptoethanol, and 0.02 M Tris, pH 7.5, and was dialyzed against the same buffer. The dialyzed fraction was loaded onto a diethylaminoethyl-Sephadex A-50 column (2 by 19 cm; Pharmacia) which was then washed with 75 ml of the same buffer. EF-G activity was then eluted with the same buffer with a linear gradient of 0.2 to 0.45 M KCl by using 300 ml each of the starting and limit buffers and collecting 7.0-ml portions. The peak fractions were pooled and concentrated by ammonium sulfate (65% saturation) precipitation and final suspension in TMS buffer. The Sephadex A-50 column method was modified from Kaziro et al. (22) and Aharnowitz and Ron (2).

Assays for EF-G and EF-Tu. EF-Tu was assayed by its ability to complement the activity of a heat-inactivated S-100 fractions containing a temperature-sensitive EF-Tu (from strain IS13) in a washed ribosome-polyuracil-polyphenylalanine-synthesizing system (11). In some assays [^{14}C]phenylalanyl tRNA and purified EF-G were incubated with purified EF-Tu, ribosomes, and polyuracil (see Table 4). EF-G activity was measured by testing the ability of fractions to complement heat-inactivated S-100 fractions containing a temperature-sensitive EF-G (from strain IS9), as described above (11).

The preparation of washed ribosomes and S-100 proteins and their heat inactivation (from strains IS9 and IS13) were as described previously (11). Polyuracil-dependent polyphenylalanine synthesis was performed as previously described (16) but in a final volume of 0.05 ml. Quantities of 15 μg of S-100 protein and 15 μg of ribosomes as ribosomal protein were added to each assay tube.

When the effect of methylkirromycin on in vitro protein synthesis was studied, appropriate dilutions of the antibiotic stock solution (10 mg/ml in 20% ethanol) were made so that the final ethanol concentration in all assay tubes, including the ones without methylkirromycin, was 2%. The methylkirromycin was kindly provided by Sidney Pestka (Roche Institute).

Gel electrophoresis. Two-dimensional isoelectric-focusing gels were run by the method of O'Farrell (30), with electrofocusing in the first dimension and sodium dodecyl sulfate (SDS)-gel electrophoresis in the second dimension. One-dimensional SDS-slab gels were run by using the second dimension system of O'Farrell. One-dimensional isoelectric-focusing slab gels used the first-dimension system of O'Farrell as modified by Ames and Nikaido (3), using riboflavin instead of ammonium persulfate to polymerize the gels and omit-

ting the prerunning step. Briefly, the first dimension, consisting of 4% acrylamide and a 4:1 ratio of pH 5 to 7-pH 3 to 10 ampholytes (LKB Instruments Inc. or Bio-Rad Laboratories), separates proteins on the basis of charge. The second dimension, SDS-10% acrylamide, distinguishes proteins on the basis of size.

SDS-slab gels were stained with 0.1% Coomassie brilliant blue in 50% ethanol–7.5% acetic acid and destained in 30% ethanol–7.5% acetic acid. The isoelectric slab gels were fixed in 50% ethanol–7.5% acetic acid for 24 h before staining and destaining as described above. In all experiments, the slab gel apparatus of Studier (36) was employed, using 1-mm spacers.

Antibiotics. Streptomycin sulfate was purchased from E. R. Squibb & Sons. Erythromycin (Ilotycin gluceptate) was obtained from Eli Lilly & Co. Fusidic acid was a gift from W. O. Godtfredson (Leo Pharmaceutical Products, Ltd.). George B. Whitfield, Jr. (The Upjohn Co.) kindly provided spectinomycin sulfate. H. Wolf (Gesellschaft für Molekularbiologische Förschung) generously gave kirromycin, and methylkirromycin (X5108) was donated by S. Pestka (Roche Institute). All other chemicals were obtained from the sources previously described (11, 16).

RESULTS

Isolation of kirromycin-resistant mutants. Five mutants of strain IS3 (Table 1) were isolated spontaneously at a frequency of approximately 10^{-7} by growth on TBAB agar containing 200 μg of kirromycin per ml. All were found to map in the same area of the *B. subtilis* chromosome by using transduction (see below). Levels of kirromycin lower than 100 μg/ml did not inhibit the growth of sensitive cells. Because these mutants, although isolated on separate petri dishes, were from the same overnight culture and could have been siblings, only one, strain IS111, was studied further. Another mutant, strain IS115, was isolated from another single colony of strain IS3 by growth on 400 μg of methylkirromycin (27) per ml. All mutants isolated were resistant to at least 400 μg of kirromycin or methylkirromycin per ml (highest levels tested), and no difference in the in vitro or in vivo effects of these two antibiotics was observed.

Transductional mapping of kirromycin resistance mutations. We have shown previously that a mutation, *ts-5*, affecting EF-Tu activity in vitro, mapped between *strA* and *spcA* (11). Figure 1 illustrates our most recent transductional map of the *cysA-lin-2* chromosomal segment. Two strains bearing kirromycin-resistant mutations; IS111 (*kir-2*) and IS115 (*kir-7*), were used as recipients for transducing bacteriophage grown on a *B. subtilis* derivative carrying *strA* and *spcA* (strain IS26).

The data in Table 2 clearly show that both *kir* markers map between *strA* and *spcA*. Other experiments, not illustrated, also show that the other *kir* markers discussed above also map between *strA* and *spcA*. To further map the *kir* mutation, transductional crosses between strains IS121 (*ery-1*) and IS111 (*kir-2*) were performed (Table 2). Although the recombination between *ery-1* and *kir-2* was relatively low, the three-factor data and the distances from two-factor analysis (summarized in Fig. 2A) unambiguously place *kir-2* proximally to *ery-1*. In these crosses, a relatively low representation of certain classes and an overrepresentation of others ("1111" and "111" classes with Cys^+ selection) were observed. We have no clear explanation for this phenomenon, but it may result from an interaction between antibiotic resistance markers. We have previously observed asymmetry in reciprocal transductional crosses in this chromosomal region (15).

It was not possible to map *kir* relative to the aminoglycoside resistance markers *nea-1*, *kan-2*, and *neo-3*, which are proximal to *ery-1* (Fig. 1), as these markers could not be introduced into *kir* recipients by transduction. We have noted previously that certain combinations of the *nea*, *neo*, and *kan* mutations with other antibiotic resistant markers lead to the death or extremely slow growth of recombinants (17).

Transformational mapping of kirromycin resistance mutations. We have shown previously that the markers *strA*, coding for ribosomal protein S12, and *ts-6*, affecting EF-G in vitro, map proximally to *ts-5*, which affects EF-Tu (11). We have also mapped *fus-1*, which determines resistance to fusidic acid, an EF-G-specific antibiotic, to the right of *strA* (17) (Fig. 1). This suggested that *kir* would map to the right (distally) of *ts-6* and *fus-1*, both EF-G mutations, and should be more closely linked to *ts-5* (EF-Tu), if it were involved with the synthesis of EF-Tu.

Because the genetic distances separating *strA* and *ts-5* are relatively small, we undertook a series of experiments involving DNA-mediated transformation to increase the level of genetic recombination in these crosses. DNA isolated from an *strA-kir-2* derivative was used to transform competent cells bearing the *fus-1*, *ts-6*, or *ts-5* markers. The data in Table 3 demonstrate that *kir-2* is distal to *ts-6*, *fus-1*, and *ts-5*. The two-factor distances in these crosses are shown in Fig. 2B.

Our results indicate that mutations affecting EF-G are to the right of *strA* and to the left of those involved with EF-Tu. At present, no additional genes have been found between *strA* and *kir-2*.

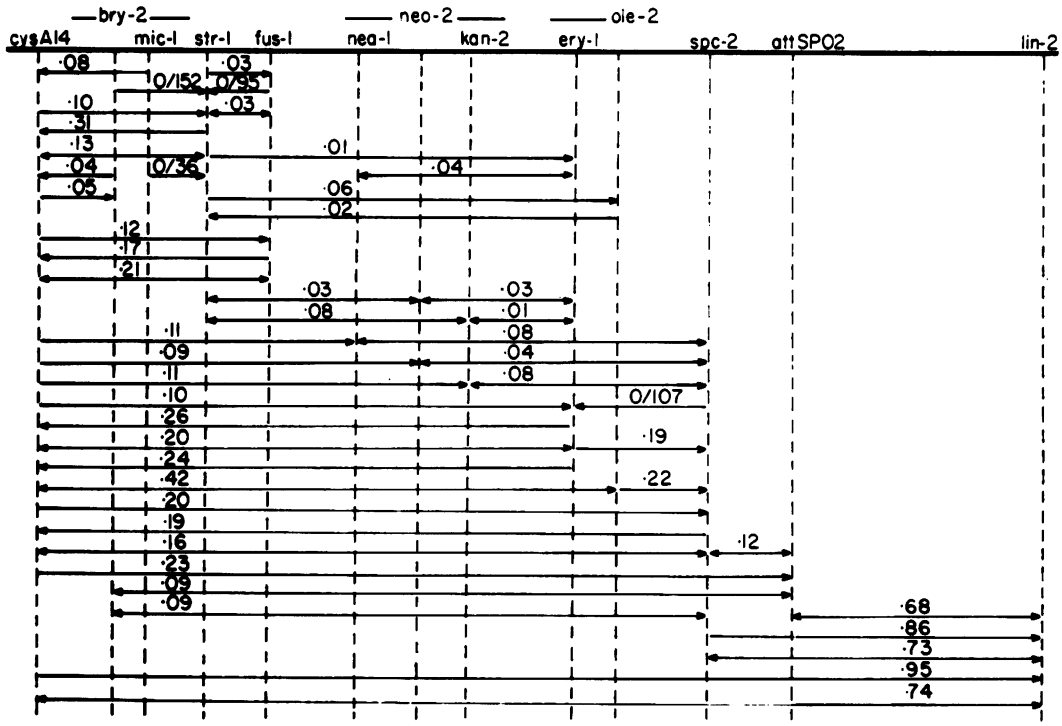

FIG. 1. *Genetic map of cysA-lin-2 segment of the B. subtilis chromosome. The order and distances have been determined from three-factor crosses utilizing B. subtilis transducing phages PBS1 and AR9 (15, 17, 35). In all cases the head of the arrow points to the recipient marker of the recombinant class. Double-headed arrows indicate that both markers were recipients in different crosses. The numbers represent fractional recombination between markers. In certain cases, no recombination was observed (e.g., 0/152). The abbreviations bry, mic, str, fus, nea, neo, kan, ery, ole, spc, and lin denote mutations conferring resistance to bryamycin (thiostrepton), micrococcin, streptomycin, fusidic acid, neamine, neomycin, kanamycin, erythromycin, oleandomycin, spectinomycin, and lincomycin, respectively (all mutations were spontaneously derived). attSPO2 is the attachment site for the lysogenic B. subtilis bacteriophage SPO2, whereas cys designates a mutation affecting the synthesis of cysteine. The map is not drawn to scale, especially in the area between attSPO2 and lin-2.*

Purification of wild-type and mutant EF-Tu. To determine whether kirromycin resistance in vitro was due to an altered EF-Tu, as has been demonstrated in *E. coli* (12, 39), we proceeded to purify EF-Tu from *B. subtilis* wild-type and isogenic *kir* strains. EF-Tu was purified from ribosome-free S-100 fractions of a wild-type strain, IS6, by ammonium sulfate precipitation and affinity column chromatography, using GDP-Sepharose columns and GDP elution as described by Jacobson and Rosenbusch (18). Figure 3A shows a typical elution pattern of EF-Tu activity from the GDP-Sepharose column. Approximately 50% of the EF-Tu activity was retained on the GDP-Sepharose column and was specifically eluted by 100 μM GDP. This fraction was more than 90% pure, as shown by SDS gels (Fig. 4, channel 5) and two-dimensional isoelectric-focusing gels (Fig. 5B). A yield of up to 10% with a 10-fold enrichment of the EF-Tu activity was routinely recovered in the GDP eluate fraction. Why a large proportion of the EF-Tu activity was not retained on GDP-Sepharose is not known as yet, but it is not due to overloading of the affinity column (unpublished data). The behavior of this fraction on Sephadex G-50 columns (Fig. 3B) suggests that it may be an EF-Tu-Ts complex (4) which would prevent binding of this molecular species to the GDP affinity column. The EF-Tu present in this fraction had the same molecular weight as that of the EF-Tu eluted by GDP (Fig. 4, channel 7). Two forms of *E. coli* EF-Tu, separable by Sephadex A-50 chromatography, have been observed (14), but it is not known whether the EF-Tu polymorphism reported here is a related phenomenon.

EF-G was purified from the nonretained fraction passing through the GDP-Sepharose column by ammonium sulfate precipitation and diethylaminoethyl-Sephadex A-50 column chro-

TABLE 2. *Transduction crosses involving kir-2 and kir-7*

Donor	Recipient	Selection	Recombinant classes *cysA*	*strA*	*kir*	*spcA*	Number recombinants	Probable order
IS26	IS115	Cys$^+$	1[a]	1	1	1	24	*kir-7*
			1	1	1	0	3	*cysA strA spcA*
			1	0	0	0	13	
		Str^r	1	1	1	1	6	*kir-7*
			0	1	1	1	6	*cysA strA spcA*
		Spc^r	1	1	1	1	6	
			0	1	1	1	21	*cysA strA kir-7 spcA*
			0	0	1	1	8	
			0	0	0	1	14	
IS26	IS111	Cys$^+$	1	1	1	1	26	*kir-2*
			1	1	1	0	2	*cys strA spcA*
			1	0	1	0	1	
			1	0	0	0	11	
		Spc^r	1	1	1	1	11	
			0	1	1	1	17	*cysA strA kir-2 spcA*
			0	0	1	1	4	
			0	0	0	1	8	
IS121	IS111	Cys$^+$	*cysA*	*kir*	*ery*			
			1	1	1		11	
			1	1	0		2	*cysA kir-2 ery-1*
			1	0	1		0	
			1	0	0		22	
		Ery^r	1	1	1		6	
			0	1	1		22	*cysA kir-2 ery-1*
			1	0	1		0	
			0	0	1		7	

[a] Donor and recipient classes are indicated by 1 and 0, respectively. Recombinant classes which did not appear among the recombinants are not shown in the four-factor crosses to save space.

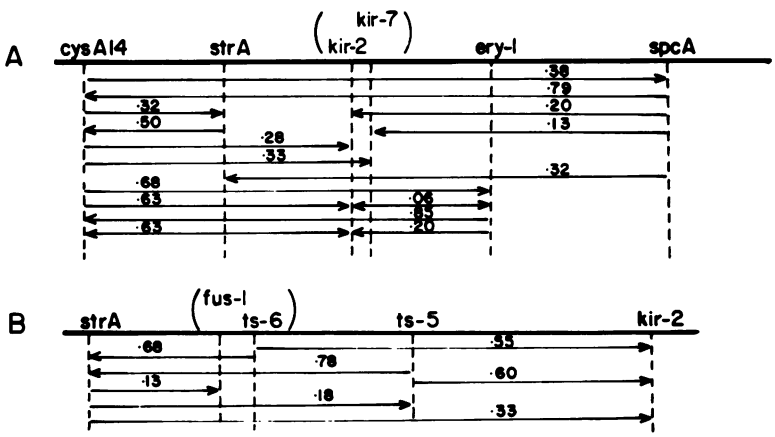

FIG. 2. *Genetic map of the cysA-spcA region. The order and distances have been determined from the transduction crosses described in Table 2 (A) and the transformation crosses described in Table 3 (B). The numbers and the arrows have the same significance as they do in Fig. 1.*

matography (Fig. 3B). The peak fractions from the column were approximately 70% pure (Fig. 4, channel 6, and Fig. 5C) and contained 25% of the initial EF-G activity, with an enrichment of up to 20-fold compared with the S-100 fraction.

Properties of EF-Tu from kirromycin-resistant and -sensitive strains. EF-Tu was purified from the isogenic strains IS3, IS111, and

TABLE 3. *Transformation crosses involving kir-2*

Donor	Recipient	Selection	Recombinant classes			Number recombinants	Probable order
			strA	*fus-1*	*kir-2*		
IS116	IS48	Str^r	1[a]	1	1	19	*strA fus-1 kir-2*
			1	1	0	4	
			1	0	1	1	
			1	0	0	4	
IS116	IS16	Ts^+	*ts-6*	*strA*	*kir-2*		
			1	1	1	1	*strA ts-6 kir-2*
			1	1	0	12	
			1	0	1	17	
			1	0	0	10	
IS116	IS13	Ts^+	*ts-5*	*strA*	*kir-2*		
			1	1	1	0	*strA ts-5 kir-2*
			1	1	0	9	
			1	0	1	16	
			1	0	0	15	
		Str^r	1	1	1	24	*strA ts-5 kir-2*
			1	1	0	7	
			0	1	1	0	
			0	1	0	7	

[a] Donor and recipient classes are indicated by 1 and 0, respectively.

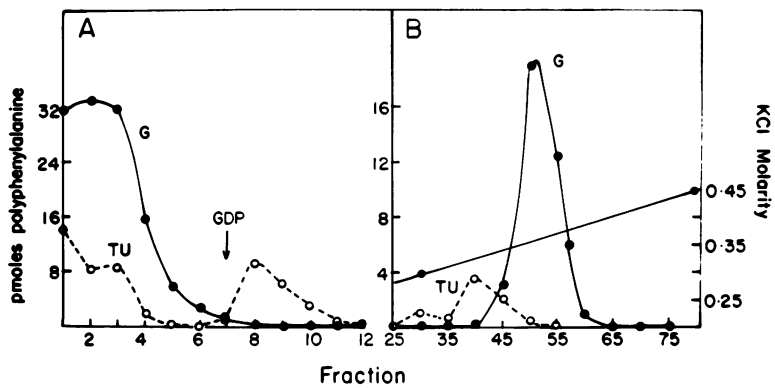

FIG. 3. *Chromatographic purification of EF-Tu and EF-G. (A) EF-Tu purification. Approximately 100 mg of protein obtained by ammonium sulfate precipitation of an S-100 fraction from strain IS6 was incubated overnight at room temperature with GDP-Sepharose in Sepharose dialysis buffer. The slurry was poured into a column, and the run-through fraction (fraction 1) was collected and washed with 5 bed volumes of the same buffer (fractions 2 to 6). EF-Tu was specifically eluted by adding 6 volumes of dialysis buffer containing 100 μM GDP (fractions 7 to 12). Each fraction was assayed for EF-G (●) and EF-Tu (○) activity by a complementation assay with a heat-inactivated S-100 fraction (strains IS9 and IS13, respectively) in a polyuracil-polyphenylalanine system. (B) EF-G purification. A 45-mg quantity of the run-through proteins from the GDP-Sepharose column (fractions 1 to 3) was fractionated on a diethylaminoethyl-Sephadex A-50 column, using a 600-ml linear 0.2 to 0.45 M KCl gradient and collecting 7.0-ml fractions. EF-G and EF-Tu activity were assayed as in (A).*

IS115 (the latter two strains bearing the *kir-2* and *kir-7* mutations, respectively), as described above, except 1 g of cells was used. The purified EF-Tu fractions were characterized by isoelectric slab gel electrophoresis (Fig. 6). The EF-Tu isolated from the *kir* mutants showed a more acidic isolectric point than did the wild-type EF-Tu. This difference was also observed in crude S-100 protein fractions.

Kirromycin has been shown to interact specif-

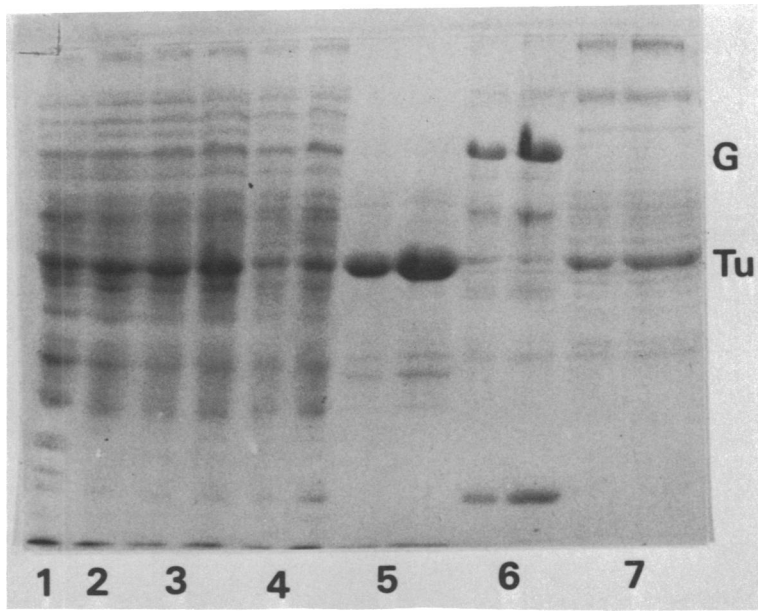

FIG. 4. *SDS gel patterns of EF-G and EF-Tu during purification. SDS-10% acrylamide slab gels were loaded with the following protein fractions from strain IS6, obtained during enzyme purification: (1) 40 μg of S-30 protein; (2) 40 μg of S-100 protein; (3) 25 and 50 μg of the 35 to 65% saturated ammonium sulfate precipitate before adding to the GDP-Sepharose column; (4) 20 and 40 μg of the run-through (fractions 1 to 3) from the GDP-Sepharose column before Sephadex A-50 fractionation; (5) 10 and 25 μg of purified EF-Tu from the GDP-Sepharose column, eluted with 100 μM GDP (fractions 8 and 9); (6) 10 and 25 μg of purified EF-G from the Sephadex A-50 column (fractions 48 to 57); (7) 10 and 25 μg of EF-Tu activity recovered from the run-through of the GDP-Sepharose column and fractionated on Sephadex A-50 (fractions 35 to 45). The purified samples were intentionally overloaded to show contaminating proteins. Samples were loaded on the top of the gel, run for 16 h at 20 V with constant voltage, and stained with Coomassie brilliant blue. The cathode and anode were at the top and bottom, respectively. The purified B. subtilis EF-G and EF-Tu migrated identically on SDS gels with E. coli EF-G and EF-Tu and calibrating protein standards (data not shown), indicating molecular weights of 83,000 and 47,000, respectively.*

ically in vitro with EF-Tu by preventing the release of EF-Tu–GDP from ribosomes (40), and mutants of *E. coli* that are resistant to kirromycin in vivo show in vitro resistance to the antibiotic at the level of EF-Tu (12, 39). We tested the effect of kirromycin in EF-Tu-dependent protein synthesis, using wild-type and mutant EF-Tu (Table 4). At the highest level of kirromycin tested here (100 μg/ml), the wild-type enzyme was inhibited by 50%, with little or no effect on the mutant EF-Tu isolated from *kir-2* and kir-7 strains. Raising the kirromycin concentration did not increase the level of inhibition in the wild-type strains (data not shown). *E. coli* EF-Tu isolated from wild-type strains is much more sensitive to kirromycin (12) than is its *B. subtilis* counterpart. Possibly, EF-Ts present in our EF-Tu assay decreased the effect of the kirromycin in the *B. subtilis* wild-type EF-Tu, as has been reported in *E. coli* (12). In any case, the level of sensitivity of the kirromycin-sensitive EF-Tu was too low to allow in vitro mixing experiments between wild-type and mutant enzymes. This type of experiment has demonstrated the in vitro dominance of kirromycin sensitivity in *E. coli* (12, 39).

Characterization of other elongation factor mutations. In the course of this work an attempt was made to characterize elongation factors isolated from other mutant strains of *B. subtilis*. We have mapped several conditional mutations adjacent to *strA* which cause EF-G to be temperature sensitive in vitro (11). Similar mutants have been studied in other laboratories (1, 23).

S-100 proteins from strains IS7, IS9, IS16 (11), IS79, and IS82 (E. Dubnau, P. Paress, and I. Smith, unpublished data), which possess temperature-sensitive EF-G activity in vitro, were analyzed on isoelectric-focusing slab gels. In addition, an S-100 fraction from strain IS13 which contains a temperature-sensitive EF-Tu (11) was also examined. No reproducible differences between wild-type EF-G or EF-Tu and these mutants were observed (data not shown).

We also analyzed EF-G from a strain bearing

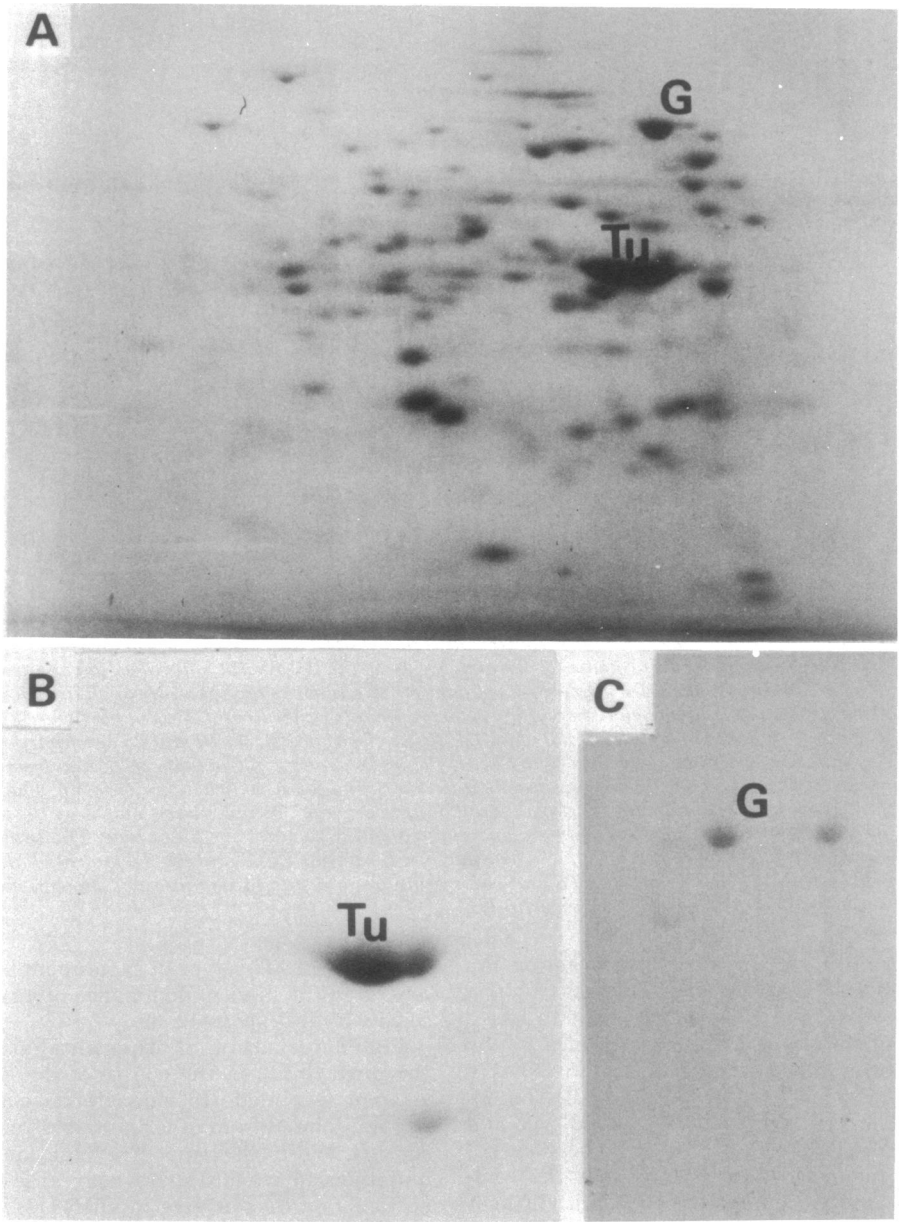

FIG. 5. *Two-dimensional isoelectric-focusing gels of elongation factors. Isoelectric-focusing gels were run with (A) 100 μg of strain IS6 S-100 total protein; (B) 50 μg of purified EF-Tu (GDP-eluted fraction from a GDP-Sepharose column); and (C) 50 μg of purified EF-G from a Sephadex A-50 column. The EF-G and EF-Tu spots in (A) were identified by comparison with several two-dimensional isoelectric-focusing gels of purified EF-Tu and EF-G similar to (B) and (C). The first dimensions were run from left (cathode) to right (anode), separating proteins on the basis of charge, with the more acidic proteins migrating further to the right. The second dimension, separating on the basis of size as in Fig. 4, was run from the top down (cathode at top, anode on bottom). The double spot in the purified EF-G gel was variable. In some purified preparations only the spot to the left was observed. [Also note the single EF-G spot in (A)].*

a fusidic acid resistance mutation, *fus-1*, which is linked to *strA*, *kir*, and *ts-5* (17). Similar mutations in *E. coli* and *B. subtilis* confer resistance in vitro to fusidic acid and map next to *strA* (6, 24, 25, 37). EF-G was purified by Sephadex A-50 chromatography from strain IS3 and

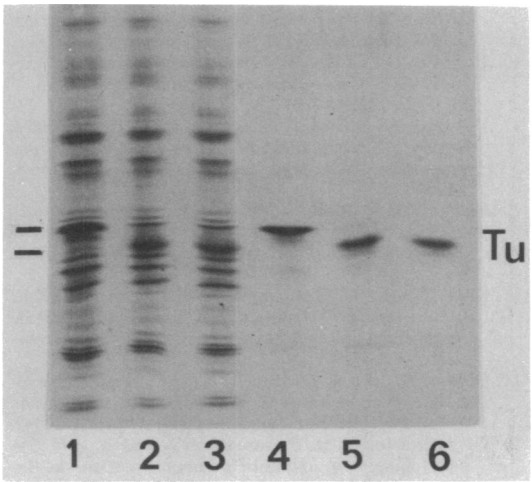

FIG. 6. *One-dimensional isoelectric-focusing slab gels of mutant and wild-type EF-Tu factors. S-100 proteins and EF-Tu from strains IS3, IS111 (kir-2), and IS115 (kir-7), purified by affinity chromatography on GDP-Sepharose columns, were analyzed on one-dimensional isoelectric-focusing slab gels. (1) Strain IS3 S-100 protein, 50 μg; (2) strain IS111 S-100 protein, 50 μg; (3) strain IS115-100 protein, 50 μg; (4) strain IS3 EF-Tu, 5 μg; (5) strain IS111 EF-Tu, 5 μg; (6) strain IS115 EF-Tu, 5 μg. Samples were loaded on the top of the gel and run with the cathode on top and the anode on bottom. Gels were stained with Coomassie brilliant blue. The upper line on the left indicates the migration of the wild-type EF-Tu, and the lower one is at the level of the mutant EF-Tu. Note that the differences are also observed in the S-100 proteins.*

a *fus-1* derivative as described above. Although no greater resistance to fusidic acid was observed with the EF-G isolated from the fusidic acid-resistant strain, slight variable migrational differences were observed on isoelectric-focusing slab gels (data not shown). The EF-G isolated from *fus* strains generally had a lower specific activity and was much more unstable than that isolated from wild-type *B. subtilis*.

DISCUSSION

Two pieces of evidence now indicate that only one functional gene for EF-Tu is found in *B. subtilis*. A temperature-sensitive mutation, *ts-5*, affecting EF-Tu in vitro, has been isolated (11). If there were two functional *tuf* genes, one would expect a loss of approximately 50% in EF-Tu activity, in vitro after heating, instead of the more than 90% loss observed. Although this could result from the unequal expression of two genes or from a mutated modifier gene, the mapping of the spontaneous kirromycin resistance mutations very close to *ts-5* also indicates that this area codes for EF-Tu. All of the EF-Tu protein from *kir* strains shows altered migration in isoelectric-focusing gels, again suggesting that only one gene is expressing in vivo. It could be argued that *kir* and *ts-5* are mutations in a modifier gene and not the structural gene for EF-Tu. This is unlikely because the kirromycin-sensitive step in EF-Tu action occurs when EF-Tu–GDP is bound to the ribosome, the antibiotic preventing its release as during normal protein synthesis (40). Yet the *ts-5* mutation causes EF-Tu to be temperature sensitive in the GDP-binding reaction in the absence of ribosomes (11). Kiromycin does not prevent GDP binding to EF-Tu (7). This suggests that different parts of the EF-Tu molecule are involved with kirromycin sensitivity and GDP or GTP binding and that *kir* and *ts-5* are mutations in the same gene, but at different sites. If two functional genes were present in *B. subtilis* as in certain strains of *E. coli* (20, 39), it would be impossible to move the *kir* marker into sensitive recipients because kirromycin sensitivity is dominant both in vivo and in vitro (12, 39). We have been able to transform *kir* into sensitive recipients either by primary selection (the formation of strain IS116 [Table 1]) or as an unselected marker (Table 3). Interestingly, one strain of *E. coli*, D22, seems to have one functional EF-Tu gene, *tufB*, and the *kir* marker in strain D22 derivatives can be transferred back to the strain

TABLE 4. *Effect of methylkirromycin on EF-Tu activity*[a]

Source of EF-Tu	Amount (μg)	Methylkirromycin conc. (μg/ml) 0	10	100
Exp't. 1				
IS3	2.3	23.7[b]	20.9	12.5
IS115	2.5	17.4	16.9	16.1
Exp't. 2				
IS3	2.3	25.4	24.1	11.2
	1.2	20.3	17.5	9.9
IS111	2.4	14.5	18.9	14.4
	1.2	16.0	16.4	15.5

[a] Each tube contained, in 50 μl, the usual ingredients for protein synthesis, using polyuracil as mRNA (11) but with 1.8 μg of purified EF-G and 50 pmol of [^{14}C]phenylalanyl tRNA (230 cpm/pmol) prepared as described previously (11); the phenylalanyl tRNA was purified with phenol and two ethanol precipitations. No S-100 protein was added to these assays. In the absence of EF-Tu, 1 to 1.5 pmol of polyphenylalanine was incorporated, over the zero time blank (80 to 100 cpm). This value is subtracted from the above data. (In the absence of EF-G and with 2 μg of EF-Tu, less than 0.5 pmol of phenylalanine was incorporated.) The samples were treated with hot trichloroacetic acid and counted after a 30-min incubation at 37°C as described previously (11).

[b] Data expressed in picomoles of polyphenylalanine synthesized in each reaction mixture.

D22 parent, but not to other *E. coli* strains (12). We assume that kirromycin sensitivity is dominant in *B. subtilis*, as in *E. coli*, although we have not been able to perform dominance tests in vivo or in vitro.

Our data do not rule out the existence of two closely linked (not separated by transformation) genes for EF-Tu. However, the spontaneous origin of the *kir* mutations at a relatively high frequency, 10^{-7}, would require that both genes would have to mutate to kirromycin resistance simultaneously at a frequency greater than 10^{-4}, an unlikely possibility.

Absolute certainty that the *kir* locus codes for EF-Tu must await primary sequence analysis of wild-type and mutant proteins. In this regard, it would be of interest to study the in vitro properties of EF-Tu isolated from *kir* and *ts-5* strains of *B. subtilis*. Single- and double-mutant enzymes could be obtained from recombinational crosses similar to those described in this communication. In this way a genetic dissection of the EF-Tu molecule could be performed to complement functional and structural studies on tryptic fragments of this enzyme (13, 19, 26).

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