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ssaD1, a Suppressor of *secA51*(Ts) That Renders Growth of *Escherichia coli* Cold Sensitive, Is an Early Amber Mutation in the Transcription Factor Gene *nusB*

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Complementation analysis of the *ssaD1* mutation, isolated as a suppressor of the *secA51*(Ts) mutation that renders growth of *Escherichia coli* cold sensitive, was used to show that *ssaD* corresponds to *nusB*, a gene known to be important in transcription antitermination. DNA sequence analysis of the *ssaD1* allele showed that it creates an amber mutation in the 15th codon of *nusB*. Analysis of the effect of different levels of NusB protein on *secA* transcription and translation suggested that NusB plays little or no role in the control of *secA* expression. Accordingly, mechanisms by which *nusB* inactivation can lead to suppression of *secA51*(Ts) and *secY24*(Ts) mutations without affecting *secA* expression need to be considered.

In an effort to identify additional genes essential for protein secretion, extragenic suppressors of *secA51*(Ts) and *secY24*(Ts) mutants that rendered the growth of *Escherichia coli* cold sensitive were isolated previously (1, 3, 13, 19, 20). These suppressor mutations have been located to a surprisingly large number of genes, most of them involved in protein synthesis (9, 14, 18, 20). It has been suggested that most of these suppressors operate indirectly by reducing the rate of protein synthesis, thereby bringing the processes of protein synthesis and secretion back into balance (9, 20). This proposal was also supported by the observation that even low levels of protein synthesis inhibitors such as chloramphenicol suppressed both temperature-sensitive and cold-sensitive *sec* mutants and resulted in increased rates of protein export under normally nonpermissive conditions (9). One suppressor of the *secY24*(Ts) mutation *ssyB63*(Cs) has been shown recently to be an insertional disruption of *nusB*, a gene important in transcription antitermination and essential for the growth of *E. coli* at low temperatures (5, 12, 22). The *ssyB63*(Cs) mutation was shown previously to cause a reduced rate of polypeptide chain elongation (20). Furthermore, an allele of *nusB*, *nusB5*(Cs), that was isolated as defective in antitermination was also shown to suppress the *secY24*(Ts) mutation similarly to *ssyB63*(Cs) (22). A suppressor of the *secA51*(Ts) mutation, *ssaD1*, was shown previously to map similarly to *ssyB* (13), raising the possibility that it may be allelic to *nusB*.

***ssaD* is allelic to *nusB*.** To determine the precise location of the *ssaD* gene, complementation analysis was performed by transforming the *ssaD1* mutant with plasmids carrying defined chromosomal segments from the relevant region. Gardel et al. (6) showed previously that pCG169 complements the cold-sensitive growth defect of the *ssaD1* mutant. We constructed two additional plasmids, pKS169 and pBB169, that contain large 5′ deletions of the chromosomal segment present on pCG169 (Fig. 1). Both of these plasmids were equivalent to pCG169 in allowing complementation. DNA sequence analysis of the chromosomal insert on pBB169 using the dideoxy method (16) showed that it contained the distal end of an open reading frame, ORF2, an adjacent open reading frame, ORF3, and the first 108 codons of *nusB*, a sequence reported previously by Taura et al. (22). To determine whether ORF3 or *nusB* was responsible for the observed complementation, pSB1208 containing solely the *nusB* gene under *lac* promoter control was used (22). Since this plasmid was also equally active in the complementation assay (Fig. 1), it was concluded that *ssaD* is allelic to *nusB* and that the truncated NusB protein is sufficient for complementation in this case.

To determine the DNA sequence alteration caused by the *ssaD1* mutation, we used the sequences flanking *nusB* reported previously by Ishii et al. (8) to design PCR primers for amplification of the entire *nusB* gene from chromosomal DNAs isolated from isogenic wild-type and *ssaD1* strains by using previously established methods (15). However, in contrast to the 0.5-kb DNA fragment predicted on the basis of the DNA sequence of Ishii et al. (8), a 0.85-kb fragment was obtained in each case (Fig. 2). The 0.85-kb DNA fragment derived from the *ssaD1* mutant was treated with T4 DNA polymerase, purified by elution after agarose gel electrophoresis, and cloned into the *Eco*RV site of pBluescript II KS$^+$ (Stratagene, La Jolla, Calif.) by using established methods (15). DNA sequence analysis of the entire *nusB* gene on the resulting plasmid revealed a single mutation, a C-to-T transition, resulting in the conversion of the 15th codon normally encoding Gln to an amber termination codon. Thus, the *ssaD1* allele is essentially a null mutation since this strain background lacks informational suppressor activity. Additional DNA sequence analysis of the region immediately downstream of *nusB* revealed the reason for the size discrepancy of the PCR fragments obtained. Comparison of our DNA sequence with that obtained by Ishii et al. (8) revealed that their sequence contains an additional 21 bp downstream of *nusB* that corresponds to a small *Sau*3A DNA fragment that must have been introduced as a cloning artifact (nucleotides 577 to 597 of their DNA sequence) (Fig. 2). We fortuitously used this incorrect sequence to design a primer (Fig. 2, primer 2) for amplification of *nusB* by PCR. Since we obtained a 0.85-kb DNA fragment instead of a 0.5-kb DNA fragment, our data indicate that either the 21-bp sequence is located 0.35 kb downstream of *nusB* or there happens to be sufficient homology in this region for annealing of the primer.

Effect of NusB levels on *secA* expression. Our results and those of Taura et al. (22) indicate that loss of *nusB* function

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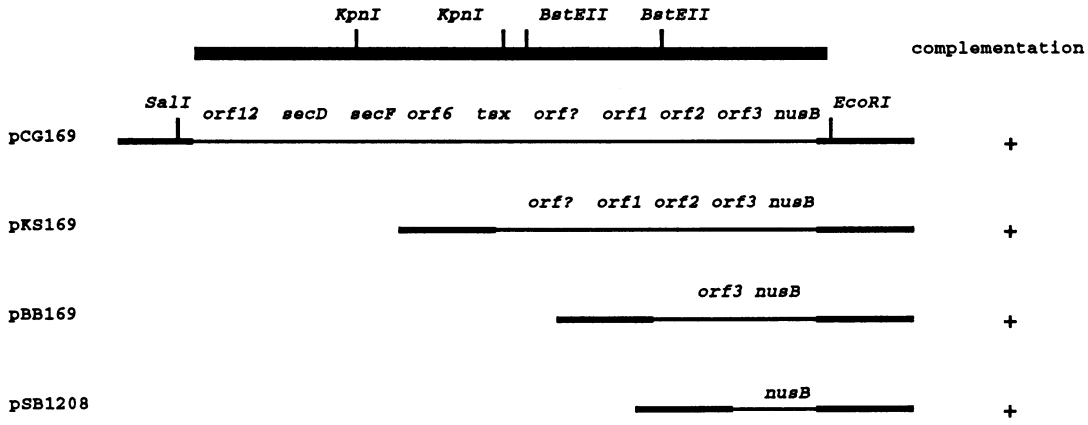

FIG. 1. *nusB* complements the *ssaD1* mutation. The chromosomal genes carried by each plasmid along with the complementation activity of the plasmid when transformed into the *ssaD1* mutant are shown. A plus (+) indicates growth and normal single-colony formation after incubation of the relevant strain on TYE-ampicillin plates at 30°C for 24 h. A negative control, the *ssaD1* mutant containing pBR322, was also included, but for the sake of simplicity, the results are not shown. pKS169 was made by deletion of the relevant chromosomal segment from pCG169 by cleavage with *Sal*I and *Kpn*I, while pBB169 was made by further deletion of pKS169 by cleavage with *Bst*EII using established methods for plasmid construction (15). pCG169 and pSB1208 have been described previously (6, 22).

allows suppression of the *secA51*(Ts) and *secY24*(Ts) mutations that reduce the function of SecA and SecY proteins, respectively. It was shown previously that the loss of *nusB* function does not elevate the rate of synthesis of SecY protein (22). It occurred to us that suppression of both of these *sec* defects may arise by a mechanism in which *nusB* inactivation leads to enhanced *secA* expression since high concentrations of SecA have been shown to suppress the protein translocation defect found normally in *secY24*(Ts) inverted inner membrane vesicles (2). Transcription of the geneX-*secA* operon occurs both by initiation immediately upstream of geneX as well as by read-through from *envA* (17). For *nusB* inactivation to cause increased transcription of *secA*, however, NusB would have to normally promote transcription termination rather than antitermination in this case. To investigate the effect of NusB concentration on *secA* expression by using strains containing *secA-lacZ* operon or protein fusions, two scenarios were examined: one in which NusB was overproduced from plasmids and one in which NusB protein was reduced because of the presence of the *ssaD1* or *nusB5* allele (4, 22). NusB protein has been shown to be undetectable in the *nusB5* mutant by Western blot (immunoblot) analysis (10, 21). Both *secA-lacZ* operon fusion and protein fusion strains showed minor differences in *secA* expression whether the *nusB* gene dosage was increased because of the presence of plasmid pBB169 or pSB1208 or the NusB protein levels were reduced drastically because of the presence of the *ssaD1* or *nusB5* allele (Table 1). An immunoblot of SecA protein from these strains gave a

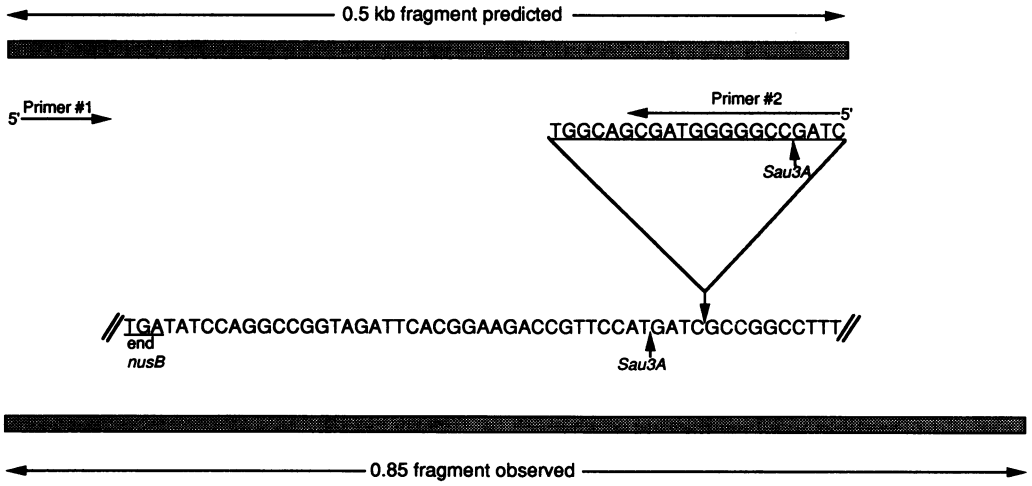

FIG. 2. Corrected DNA sequence distal to *nusB*. A small portion of our DNA sequence distal to *nusB* along with the location of an additional 21 nucleotides reported originally by Ishii et al. (8) (given above our sequence) is shown. The termination codon of *nusB* is underlined, and the locations of two *Sau*3A sites flanking the 21-nucleotide region are shown. The sizes and locations of two DNA fragments predicted or observed for PCR amplification from two primers, primer 1 and primer 2, are also depicted. The corrected nucleotide sequence has been assigned GenBank accession number U09348.

TABLE 1. Effect of NusB on *secA* expression

Fusion type	Strain[b]	β-Galactosidase activity[c]
secA-lacZ operon fusion	DO308	1,732 ± 0
	DO308(pBR322)	1,734 ± 0
	DO308(pBB169)	1,652 ± 41
	DO308(pSB1208)	1,126 ± 33
	DR308	1,887 ± 3
	DR310	1,586 ± 14
secA-lacZ protein fusion	MM171.2	200 ± 7
	MM171.2(pBR322)	246 ± 36
	MM171.2(pBB169)	258 ± 37
	MM171.2(pSB1208)	246 ± 7
	DR312	186 ± 7

[a] Strains were subcultured in duplicate into Luria broth (10 g of Bacto Tryptone, 5 g of yeast extract, 5 g of NaCl per liter) supplemented with 20 μg of ampicillin per ml where appropriate and grown at 37°C until the mid-logarithmic phase of growth, at which time they were placed onto ice. Dilutions of the cultures were plated on TYE (10 g of Bacto Tryptone, 5 g of yeast extract, 8 g of NaCl, 15 g of Bacto Agar per liter) and TYE-ampicillin plates to score for plasmid loss, which was less than 2% in plasmid-containing cultures.

[b] DO308 [F⁻ Φ(*secA-lacZ*)*mf10 leu*::Tn*5* Δ*lacU169 araD136 relA1 rspL150 thi deoC7 flbB5301 ptsF25* (λPR9)] and MM171.2 [F⁻ Φ(*secA-lacZ*)*f181*(Hyb) Δ*lacU169 araD136 relA1 rspL 150 thi deoC7 flbB5301 ptsF25* (λPR9)] containing a *secA-lacZ* operon fusion or protein fusion, respectively, at the normal *secA* locus and an additional copy of the geneX-*secA* operon on λPR9 have been described previously (17). To construct DR308, the *nusB5 zai*::Tn*10* region was transduced from YN2416 (22) into DO308 by P1 transduction and tetracycline-resistant transductants were selected at 37°C and screened for cold sensitivity of growth at 30°C. In a similar manner, DR310 and DR312 were constructed by transducing *ssaD1 tsx*::Tn*10* into DO308 or MM171.2, respectively, except that tetracycline-resistant transductants were selected at 42°C and screened for cold sensitivity of growth at 30°C.

[c] β-Galactosidase activity, expressed as means ± standard deviations, was determined by the method of Miller (11).

similar result (data not shown). Taken together, these results indicate that NusB levels have little or no effect on *secA* expression. Thus, we conclude that the mechanism of *secA51*(Ts) and *secY24*(Ts) suppression by *nusB* inactivation does not appear to involve the production of additional SecA protein.

The largest class of extragenic suppressors of *secA51*(Ts) and *secY24*(Ts) mutations lies in components of the translational apparatus (9, 14, 18, 20). They appear to function by reducing the rate of protein synthesis, thereby bringing the rates of protein synthesis and secretion into accord (9, 20). The *ssyB63*(Cs) mutation was shown to cause a reduced rate of polypeptide chain elongation (20). Presumably, a similar scenario also holds for the *ssaD1*(Cs) mutation, since both alleles inactivate *nusB* and suppress protein secretion defects. The simplest model that may be envisioned to explain suppression of the *secA51*(Ts) and *secY24*(Ts) defects is one where inactivation of *nusB* causes a reduced rate of polypeptide chain elongation, thereby allowing an increased kinetic window for cotranslationally secreted proteins to interact with the compromised secretion apparatus as well as reducing the rate at which preproteins must be exported from the cytoplasm. However, since *nusB* controls transcription termination in a global fashion (7, 22), other models for *nusB* suppression of the observed secretion defects also need to be considered. For example, it is possible that *nusB* inactivation leads to altered expression of a *sec* gene(s) other than *secA* or *secY* or to reduction in the expression of particular genes encoding abundant envelope proteins. Careful measurements of a variety of parameters (e.g., translation rate, secretion rate, and amount) should allow discrimination between these various suppression mechanisms.

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