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Characterization of the *sppA* Gene Coding for Protease IV, a Signal Peptide Peptidase of *Escherichia coli*

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The *sppA* gene codes for protease IV, a signal peptide peptidase of *Escherichia coli*. Using the gene cloned on a plasmid, we constructed an *E. coli* strain carrying the ampicillin resistance gene near the chromosomal *sppA* gene and an *sppA* deletion strain in which the deleted portion was replaced by the kanamycin resistance gene. Using these strains, we mapped the *sppA* gene at 38.5 min on the chromosome, the gene order being *katE-xthA-sppA-pncA*. Although digestion of the signal peptide that accumulated in the cell envelope fraction was considerably slower in the deletion mutant than in the *sppA*$^+$ strain, it was still significant, suggesting the participation of another envelope protease(s) in signal peptide digestion.

A protein that is to be exported across the cytoplasmic membrane of a bacterium is synthesized as a large precursor containing a signal peptide in its amino terminus (3). The processing of this precursor involves two sequential events. First, the signal peptide is removed from the precursor through an endo-type cleavage catalyzed by a signal peptidase associated with the cytoplasmic membrane. Two signal peptidases, lipoprotein signal peptidase (12, 16, 34, 37) and leader peptidase (8, 30, 38), in *Escherichia coli* have been well characterized. The second event is the digestion of the cleaved signal peptide by another membrane protease(s), which is called signal peptide peptidase (13) or signal peptide hydrolase (25). We previously demonstrated that protease IV (27, 28), a membrane protease of *E. coli*, specifically hydrolyzes the signal peptide that accumulates in the cytoplasmic membrane as the result of translocation of the major lipoprotein (14). We then cloned the gene coding for protease IV (*sppA*) and characterized the structure of the gene and the encoded protease (15).

With the cloned gene, it was possible to map the gene locus on the chromosome. It was also possible to construct chromosomal deletion mutants. In the present work, we determined the position of the *sppA* gene on the *E. coli* chromosome. We also studied the role of protease IV in signal peptide digestion by using an *sppA* deletion mutant.

MATERIALS AND METHODS

Bacteria, plasmids, and bacteriophage. The *E. coli* strains, plasmids, and bacteriophage used in this study are listed in Table 1.

Media. LB medium (24) containing 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), and 5 g of NaCl per liter and M9–0.2% glucose medium (24) were used. For solid cultivations, the media were supplemented with 1.5% agar. When required, the following were added to the media: amino acids (25 μg/ml each), nucleotides (25 μg/ml each), thiamine hydrochloride (0.1 μg/ml), ampicillin (50 μg/ml), tetracycline (15 μg/ml), and kanamycin (25 or 12.5 μg/ml).

DNA techniques. Restriction endonucleases and bacteriophage T4 ligase were obtained from Takara Shuzo Co. [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol) and a nick translation kit were obtained from Amersham International. The conditions used for the reactions with these enzymes were those proposed by the manufacturers. Southern blotting was carried out as described previously (21), and the blots were hybridized with a ^{32}P-labeled probe (10^6 cpm/ml) at 37°C in a deionized solution of 50% formamide–5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). DNA fragments used as probes were labeled with [α-^{32}P]dCTP by means of nick translation. Other general manipulations, including the preparation of plasmids and chromosomal DNAs, ligation, and transformation, were performed as described previously (19).

Genetic procedures. Interrupted mating and P1 *kc*-mediated generalized transduction were carried out as described by Miller (24). When strains in which a plasmid carrying a temperature-sensitive replicon [*ori*(Ts)] was integrated in the chromosome were used, cultivations were carried out at 42°C (1).

Scoring and selection of mutant strains. *aroD* strains were selected on the basis of their requirement for 0.1 μg of shikimic acid and 25 μg each of tryptophan, tryosine, and phenylalanine per ml (31). *pps* strains were scored as to their inability to grow on M9 agar plates supplemented with 0.8% sodium pyruvate (4). Temperature-sensitive *pheTS* [*pheTS*(Ts)] strains were scored as to their failure to grow at 42°C on LB agar plates. The *katE* mutation was scored in the *katG* cells as to their inability to evolve bubbles with a drop of 30% H_2O_2 (17, 18). *pncA* strains were scored as to their characteristics of growth on M9-glucose medium supplemented with 0.1 mM nicotinic acid but not on that supplemented with 0.1 mM nicotinamide (9). *fadD* strains were scored as to their inability to grow on M9–0.1% sodium palmitate medium (26).

Other methods. Labeling of *E. coli* B with [2,3-^{3}H]leucine, preparation of cell envelope fractions, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography were carried out as described previously (12). Protease IV activity was assayed with *N*-benzyloxycarbonyl-L-valine-β-naphthyl ester as a substrate as described previously (15).

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TABLE 1. Bacterial strains, plasmids, and bacteriophage used

Strain, plasmid, or bacteriophage	Genotype	Source or reference
Strains		
E. coli K-12		
YO160*recA*	*F⁻ thi rel rspL ompC envZ recA* Φ(*ompF -lacZ*)	21
CSH62	HfrH *thi*	CGSC[a]
JE1011	F⁻ *thr leu trp his thy ara lac gal xyl mtl rspL thi*	20
PK191	Hfr *pro lac thi*	CGSC
AB1360	F⁻ *aroD his argE thi proA lacY galK mtl xyl tsx*	CGSC
KI3406	HfrC *man pps lpp pheTS* (Ts)	K. Inokuchi[b]
K63	F⁺ *man fadD his gel*	CGSC
MC4100	F⁻ *lacU169 araD rpsL relA thi flbB*	5
DF920	HfrC *fhuA22 ompF627 relA1 pit-10 pfkB20*::Tn*10 spoT1*	CGSC; 7
BW9051	F⁻ *thr-1 leuB6 proA2 pps-2 pheS11 xthA1 his-4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44*	CGSC; 23
BW9109	F⁻ *thr-1 ara-14 leuB6 lacY1 tsx-33* Δ(*gpt-proA*) *62 supE44 galK2 rac* Δ(*xthA- pncA*) *90 hisG4 rfbD1 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1* λ⁻	CGSC; 32
UM1	F⁻ *lacY rpsL thi-1 katE1 katG14*	17
TKS-1	F⁻ *pncA1 nadB4 supE44*	CGSC
TA001	CSH62, but pQ005 integrant	This study
TA002	PK191, but pQ005 integrant	This study
TA301	BW9051, but Kmʳ- Δ*sppA*	This study
TA0031	TA301, but pQ0081 integrant	This study
TA0081	BW9051, but pQ005 integrant	This study
E. coli B		
Wild type		Laboratory stock
TB0015	Kmʳ-Δ*sppA*	This study
Plasmids		
pSI001	Apʳ; cloned gene, *sppA*	15
pSI008	Apʳ; cloned gene, *sppA*	This study
pMAN031	Apʳ *ori*(Ts)	22
pQ005	Apʳ *ori*(Ts); cloned gene, *sppA*	This study
pSI024	Apʳ *ori*(Ts); cloned gene, Kmʳ-Δ*sppA*	This study
pLC10-4	ColE1; cloned gene, *xthA*	6, 29
pQ0081	Apʳ *ori*(Ts); cloned gene, truncated *xthA* (*xthA'*)	This study
pUC19	Apʳ; cloned gene, truncated *lac* (*lacPO, Z'*)	35
pSY343	Kmʳ; runaway replicon	36
pSI029	Apʳ Kmʳ *ori*(Ts)	This study
Bacteriophage	Pl *kc*	Laboratory stock

[a] CGSC, *E. coli* Genetic Stock Center.
[b] Unpublished data.

RESULTS

Integration of the ampicillin resistance (Apʳ) gene near the *sppA* gene on the chromosome. As the first step in genetic mapping of the *sppA* gene, we introduced a drug resistance marker near the *sppA* gene on the *E. coli* chromosome by means of a plasmid integration technique (1, 10, 22). Plasmid pQ005, which carries *ori*(Ts), Apʳ, and the *sppA* gene, was constructed (Fig. 1A). This plasmid cannot replicate at 42°C. Two Hfr strains, CSH62 and PK191, were then transformed with pQ005. Of about 10^4 transformants, 1 was able to grow at 42°C in the presence of ampicillin in the *recA*⁺ background. When a *recA* strain was used, the frequency of appearance of the Apʳ transformants was less than 10^{-7} at 42°C. These results indicate that the *recA*-dependent homologous recombination shown in Fig. 2A took place in the *sppA* region between pQ005 and the host chromosome. The integrants obtained, which were assumed to carry two *sppA* genes with the Apʳ gene in between, were stable as long as they were grown at 42°C. The integrants thus constructed from CSH62 and PK191 were named TA001 and TA002, respectively, and used in the following genetic studies.

Genetic mapping of the Apʳ gene integrated near the *sppA* gene. The location of the Apʳ gene integrated near the *sppA* gene on the chromosome of these Hfr strains was first investigated by means of the interrupted-mating technique with JE1011 as the recipient. When TA001 (HfrH; clockwise from 98.5 min) was used as the donor, the Apʳ marker was concurrently introduced with the *his* marker located at 44 min. On the other hand, no Apʳ conjugant was formed in 20 min when TA002 (Hfr; clockwise from 42 min) was used as the donor. These results indicate that the Apʳ gene is located before but close to 42 min on the chromosome.

The gene order between 37 and 42 min on the *E. coli* chromosome is *aroD-pps-pheTS-katE-xthA-pncA-fadD* (2). P1 *kc*-mediated generalized transduction was then carried out with these genetic markers. The results shown in Table 2 indicate that the Apʳ gene is most probably located beween the *katE* and *pncA* genes, possibly close to the *xthA* gene. A strain from which the *xthA-pncA* region is deleted is known (32). Consistent with the above-described results, this strain, BW9109, did not exhibit protease IV activity (data not shown).

Construction of an *sppA* deletion mutant. For fine mapping of the *sppA* gene and for studying the physiological function of protease IV, we constructed an *sppA* deletion mutant. First, pSI024, in which the 5′ portion of the *sppA* gene is replaced by the intact kanamycin resistance (Kmʳ) gene, was constructed (Fig. 1B). The total length of the truncated *sppA* gene plus the Kmʳ gene in this plasmid is approximately the same as that of the intact *sppA* gene. BW9051 was then transformed with pSI024, followed by selection for kanamycin resistance at 42°C. As many as 99% of the Kmʳ transformants were ampicillin resistant as well, suggesting that these are the integrants shown in Fig. 2B. About 1%, however, were ampicillin sensitive, suggesting that the second recombination shown in Fig. 2B took place in these cells. The recombinants thus constructed were assumed to be *sppA* deletion mutants carrying the Kmʳ gene upstream of the truncated *sppA* gene.

One of the recombinants, TA301, was subjected to further studies. TA301 did not exhibit protease IV activity at all, whereas the protase IV activity of TA0081, which is assumed to carry two *sppA* genes, was twice as high as that of BW9051, which carries one *sppA* gene (Fig. 3). To confirm the deletion of the *sppA* gene, we digested the chromosomal DNA from the wild-type strain, BW9051, and that from TA301 with *Sal*I and then analyzed the digests by Southern blotting with the 3.4-kilobase (kb) *sppA* gene-carrying *Hin*dIII fragment prepared from pQ005 as a probe (Fig. 4). The DNA from the wild type exhibited a 3.4-kb band, whereas no such band was detected with the TA301 DNA. Instead, the TA301 DNA exhibited 2.3- and 1.1-kb fragments that reacted with the Kmʳ gene-carrying probe. These results

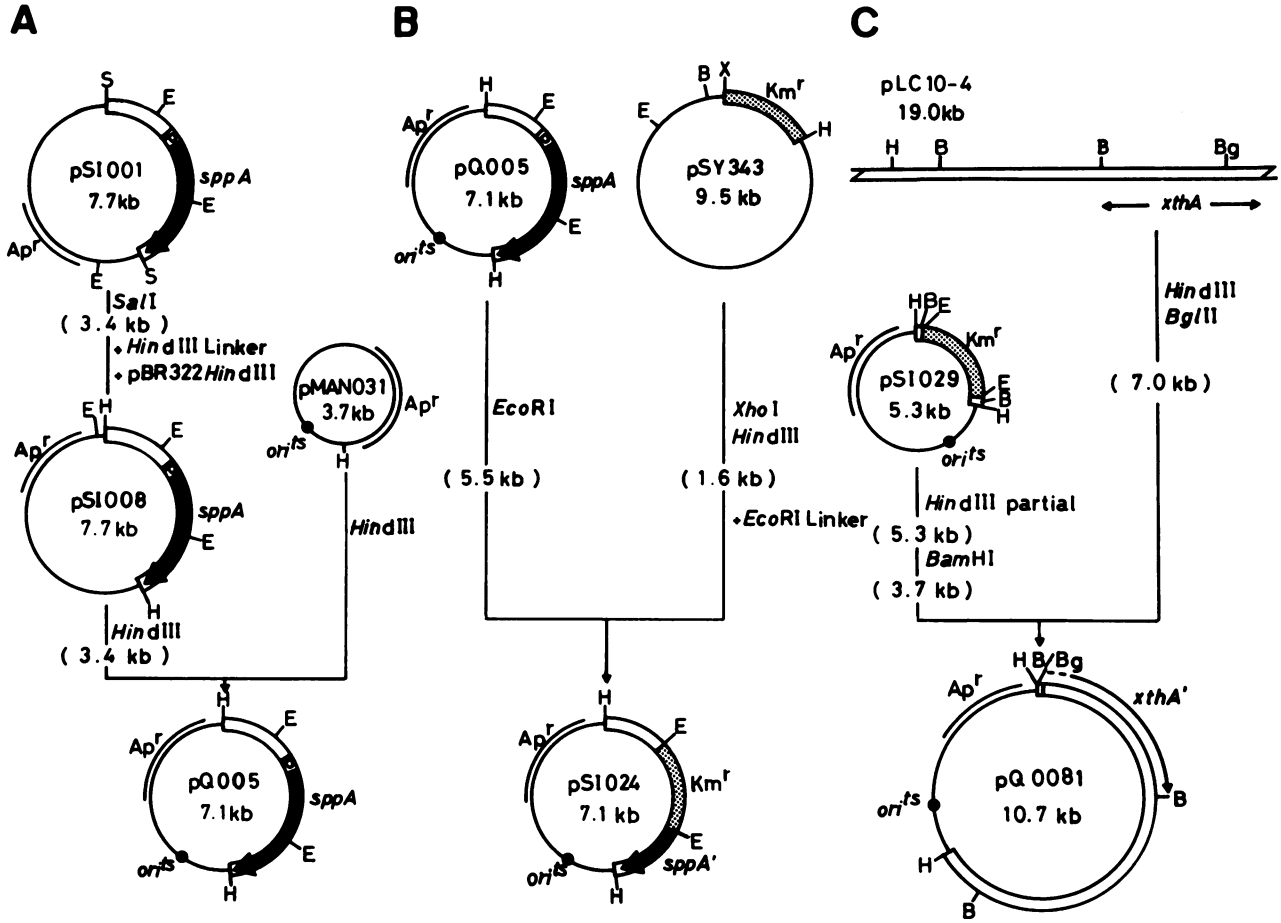

FIG. 1. Construction of plasmids pQ005 (A), pSI024 (B), and pQ0081 (C). Symbols: ▭, chromosomal DNA; ▬, Km^r gene; ▯ and ➡, promoter and protein-coding regions of the *sppA* gene, respectively. *sppA'*, Truncated *sppA* gene. Restriction endonuclease cleavage sites are denoted by the following abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I. The size of each DNA fragment formed as a result of digestion with an endonuclease is shown in parentheses. Plasmid pSI029 was constructed from *Hin*dIII-digested pMAN031, the 1.6-kb *Eco*RI fragment of pSI024 carrying the Km^r gene, and two 51-base-pair *Hin*dIII-*Eco*RI fragments (multilinker fragments) of pUC19.

confirmed the DNA structure for TA301 shown in Fig. 2B. Hereafter, the region in which the intact Km^r gene is connected to the truncated *sppA* gene will be referred to as Km^r-Δ*sppA*.

Fine mapping of the *sppA* gene with the *sppA* deletion mutant. The *sppA* gene was mapped around the *katE-xthA-pncA* region (Table 2). Using the Km^r-Δ*sppA* strain, we carried out three-factor transductional crosses. Since exonuclease III coded for by the *xthA* gene was difficult to assay (23), a strain carrying the Ap^r gene in place of the *xthA* gene (29) was constructed and used. For this purpose, pQ0081 carrying the Ap^r gene just upstream of the truncated *xthA* gene was first constructed (Fig. 1C). The Ap^r-*xthA* region was then integrated into the chromosome of TA301, and an integrant, TA0031, was isolated by the same procedure as that shown in Fig. 2A for the *sppA* gene.

The results of the transduction experiments summarized in Table 3 indicate that the *sppA* gene is located between the *xthA* and *pncA* genes (Fig. 5). When the Wu equation (33) was used, the map position of the *sppA* gene was calculated to be 38.5 min on the basis of the data in Table 3 and the map positions estimated for *pps*, *pfkB*, *katE*, and *xthA* (2).

Effect of the *sppA* deletion on signal peptide digestion. The existence of the *sppA* deletion strain clearly indicates that protease IV is not essential for *E. coli* cells in terms of viability. The effect of the deletion on signal peptide digestion was then studied. This was done by using the wild-type *E. coli* B strain, since this strain efficiently accumulates the signal peptide-carrying precursor of the lipoprotein in the presence of globomycin (11), and hence the accumulation of the signal peptide as a processing product of the precursor was assumed to be easily studied in this strain.

The Km^r-Δ*sppA* region was transferred from TA301 to *E. coli* B by transduction with Km^r as the selected marker. One of the transductants was named TB0015. TB0015 did not exhibit protease IV activity at all. Envelope fractions in which the precursor of the major lipoprotein accumulated were then prepared from wild-type B and TB0015 cells grown in the presence of globomycin as described previously (12). When the envelope fractions were incubated after the removal of globomycin by washing, the precursor was processed to the mature lipoprotein. In the presence of antipain, the processing resulted in the accumulation of the signal peptide as described previously (13) (Fig. 6A). When the envelope fractions of TB0015 ws used, an appreciable amount of a peptide accumulated at the position corresponding to that of the signal peptide, even in the absence of antipain (Fig. 6A). When globomycin was added again to the

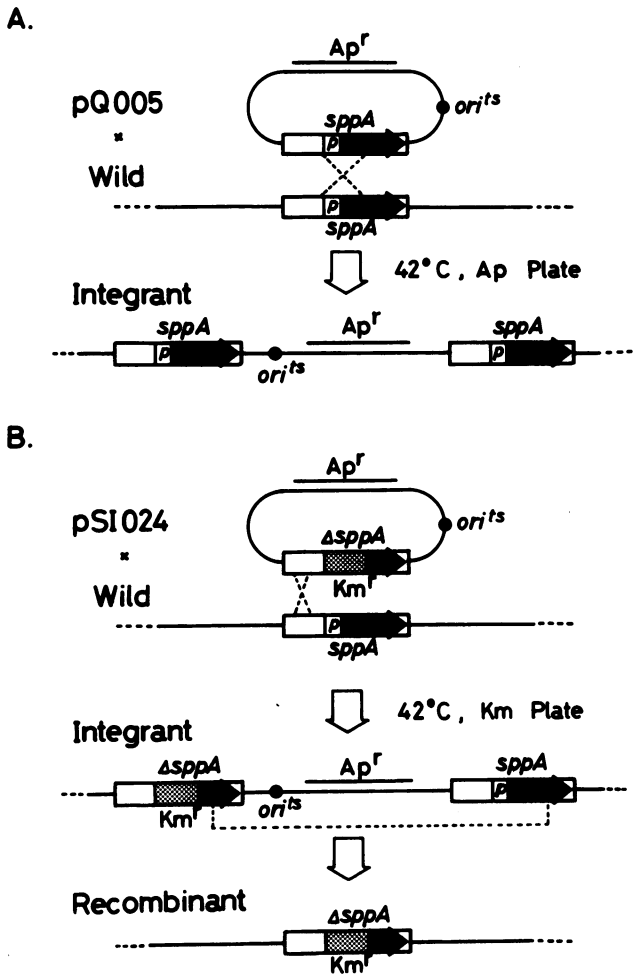

FIG. 2. Manipulation of the *sppA* gene by means of homologous recombination between plasmids and the chromosome. (A) An *sppA*$^+$ strain, BW9051, was transformed with pQ005 carrying the *sppA* gene, the Ap^r gene, and *ori*(Ts) and then incubated at 42°C on LB agar plates containing ampicillin. Plasmid integration into the chromosome through homologous recombination took place in the *sppA* region. The Ap^r marker was preserved on the chromosome of the integrant. (B) Plasmid pSI024 carries the truncated *sppA* gene (Δ*sppA*) connected to the Km^r gene, the Ap^r gene, and *ori*(Ts). An *sppA*$^+$ strain, BW9051, was transformed with pSI024 and then incubated at 42°C on LB agar plates containing kanamycin. An integrant carrying the Km^r and Ap^r markers was obtained as a result of a single recombination. A recombinant carrying the Km^r-Δ*sppA* region was obtained as a result of two successive recombinations. See legend to Fig. 1 for explanation of symbols.

incubation mixture to inhibit the cleavage of the signal peptide, no accumulation of this peptide was observed (data not shown), indicating that this was the signal peptide that was cleaved from the precursor lipoprotein. Although such an accumulation was also observed with the wild-type strain, the amount was significantly smaller.

The difference in the amount of signal peptide accumulation between the wild-type strain and TB0015 was more prominent when Triton X-100-treated envelope fractions were used as the enzyme sources (Fig. 6B). These results indicate that (i) although protease IV coded for by the *sppA* gene indeed plays a role in signal peptide digestion, it is not the only protease that is responsible for digestion in the cell envelope, and (ii) another protease(s), which is sensitive to Triton X-100, is also suggested to be involved in signal peptide digestion in the cell envelope.

TABLE 2. Cotransduction with the Ap^r gene integrated near the *sppA* gene[a]

Recipient	Unselected marker	Cotransductional frequency (%) with the Ap^r gene	No. of transductants tested
AB1360	*aroD*	9	103
KI3406	*pps*	15	100
KI3406	*pheTS*(Ts)	28	100
UM1	*katE*	48	100
TKS-1	*pncA*	40	100
K63	*fadD*	0	96

[a] TA001 was used as the donor. The P1 *kc* lysate was prepared at 42°C, and transductants were selected on LB agar plates containing 50 μg of ampicillin per ml.

DISCUSSION

In this work, we determined the map position on the *E. coli* chromosome of the *sppA* gene, which codes for protease IV, a signal peptide peptidase in the cytoplasmic membrane. Neither genes conferring protein secretion nor ones coding for proteases have been mapped in the vicinity.

Since no efficient selection method was available for the *sppA* gene, we used the Km^r gene inserted near the truncated *sppA* gene as the selection marker for the *sppA* gene. The size of the inserted DNA fragment was approximately the same as that deleted from the *sppA* gene. Therefore, the replacement should not have affected the mapping of the *sppA* gene position. We also adopted a similar technique for transductional mapping of the *xthA* gene, which codes for exonuclease III, which is difficult to assay accurately. This was done by inserting an Ap^r gene-carrying plasmid adjacent to the *xthA* gene by means of homologous recombination. In

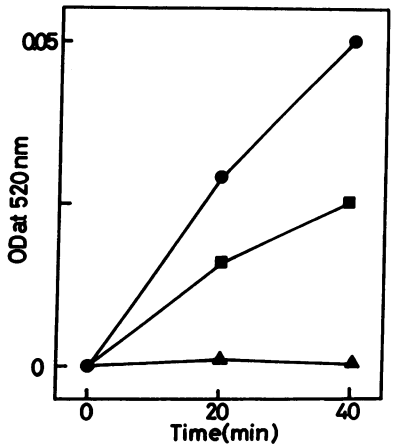

FIG. 3. Protease IV activity in the *sppA* mutants. Each strain was cultured at 42°C overnight in LB medium and then harvested. The cell envelope fraction was prepared, and the activity was assayed as described previously (15). To the envelope fraction (100 μg of protein), preincubated in 1.0 ml of 0.2% Triton X-100–10 mM Tris hydrochloride (pH 8.0) for 10 min, was added 10 μl of *N*-benzyloxycarbonyl-L-valine-β-naphthyl ester (10 mg/ml in dioxane), and incubation was carried out for the indicated times at 37°C. After the addition of Fast Garnet GBC (final concentration, 100 μg/ml), the reaction mixture was extracted with an equal volume of 1-butanol, and the optical density (OD) of the butanol layer was measured at 520 nm. Symbols: ●, TA0081 (two *sppA* genes); ■, BW9051 (one *sppA* gene); ▲, TA301 (*sppA* gene deleted).

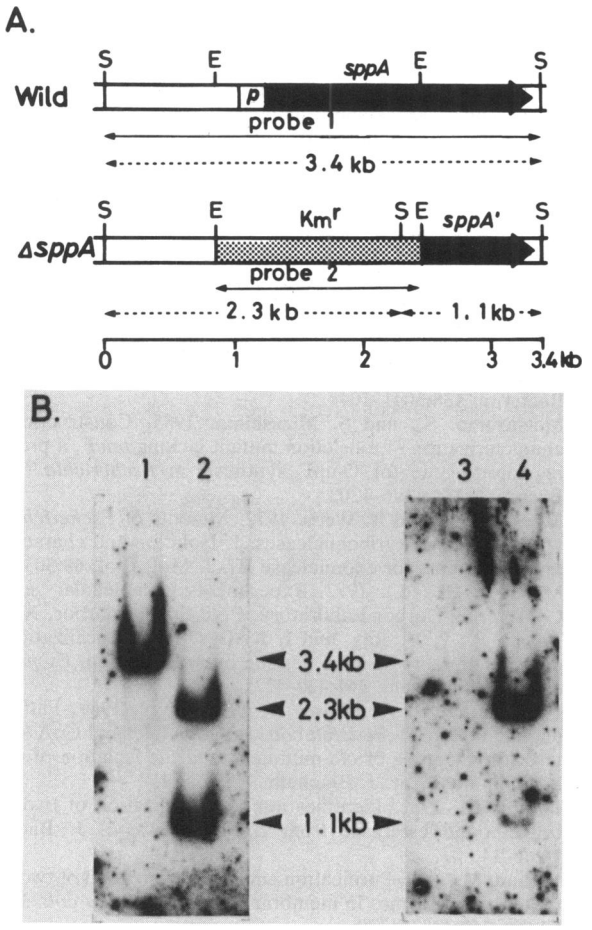

FIG. 4. Southern blot analysis of the *sppA* chromosomal deletion. (A) The chromosomal structures of the wild-type strain, BW9051, and the *sppA* deletion mutant, TA301, are shown. The symbols are the same as in Fig. 1. The 3.4-kb *Hin*dIII fragment of pQ005 and the 1.6-kb *Eco*RI fragment of pSI029 were used as probes 1 and 2, respectively. The regions corresponding to the probes are shown by the thin arrows. The sizes of the *Sal*I frgments formed as a result of *Sal*I digestion are also shown, in reference to panel B. (B) Chromosomal DNAs from BW9051 (lanes 1 and 3) and TA301 (lanes 2 and 4) were digested with *Sal*I and then subjected to Southern blot analysis as described in the text. Probe 1 (lanes 1 and 2) and probe 2 (lanes 3 and 4) were used. The sizes of the hybridized fragments are indicated by the arrowheads.

this case, however, the size of the inserted plasmid DNA was 10.7 kb, which corresponds to about 0.3 min on the *E. coli* chromosome map. Although the insertion did not affect the gene order deduced from the results of the cotransduction experiments shown in Table 3, the frequency of cotransduction must have been affected by the insertion. For estimation of the *sppA* gene position using the Wu's equation, therefore, the transduction data for cells carrying the insertion of this type were not used.

The results shown in Fig. 6 suggest that although protease

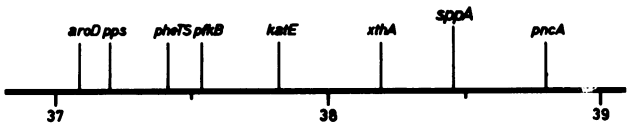

FIG. 5. Genetic map with the *sppA* gene oriented relative to nearby genes on the *E. coli* K-12 chromosome. The gene locations are based on the 100-min map (2).

TABLE 3. Three-factor transductional crosses

Donor and marker used	Recipient and marker used	Selected marker	Unselected marker	
			Class	No. (%)
TA301 Km^r (*sppA*) *pps*	DF920 Tc^{ra} (*pfkB*)	Km^r	*pps*$^+$ Tc^r	84 (85)
			pps$^+$ Tc^s	9 (9)
			pps Tc^s	5 (5)
			pps Tc^r	1 (1)
TA301 Km^r (*sppA*) *pps*	UM1 *katE*	Km^r	*pps*$^+$ *kat*	75 (68)
			pps$^+$ *kat*$^+$	32 (29)
			pps kat$^+$	3 (3)
			pps kat	0 (0)
TA0031^b Km^r (*sppA*) Ap^r (*xthA*)	UM1 *katE*	Km^{rc}	*kat* Ap^s	16 (8)
			kat Ap^r	43 (22)
			kat$^+$ Ap^r	133 (70)
			kat$^+$ Ap^s	0 (0)
TA0031^b Km^r (*sppA*) Ap^r (*xthA*)	UM1 *katE*	Ap^{rc}	*kat* Km^s	35 (12)
			kat$^+$ Km^s	51 (17)
			kat Km^r	83 (28)
			kat$^+$ Km^r	129 (43)
TA0031^b Km^r (*sppA*) Ap^r (*xthA*)	TKS-1 *pncA*	Ap^{rc}	Km^s *pnc*	12 (30)
			Km^r *pnc*	12 (30)
			Km^r *pnc*$^+$	16 (40)
			Km^s *pnc*$^+$	0 (0)

[a] Tc^r, Tetracycline resistance.
[b] P1 *kc* lysates were prepared at 42°C.
[c] Transduction and selection were performed at 42°C.

IV coded for by the *sppA* gene plays a role in signal peptide digestion in the cell envelope, another protease(s), possibly a Triton X-100-sensitive one(s), is involved as well. Ichihara et al. (14) showed that protease V in the envelope was able to digest the signal peptide, although it also digested many envelope proteins rather nonspecifically. This protease is Triton X-100 resistant, however. Participation of proteases

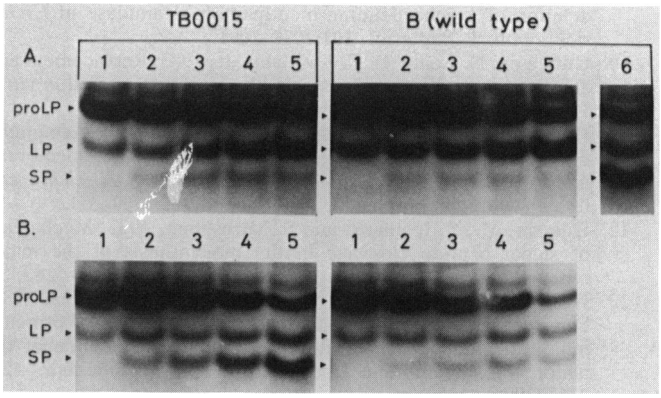

FIG. 6. Kinetic analysis of signal peptide accumulation in the cell envelope of the *sppA* deletion mutant. [^{3}H]leucine-labeled envelope fractions in which the precursor of the major lipoprotein accumulated were prepared from *E. coli* B (wild type) and TB0015 (Δ*sppA*) as described previously (9). The envelope fractions (equivalent to 0.5 ml of the culture) were then incubated for 0, 5, 10, 20, and 40 min (lanes 1, 2, 3, 4, and 5, respectively) in 10 mM sodium phosphate buffer (pH 7.1) in the absence (A) or presence (B) of 1% Triton X-100. Lane 6 is a sample incubated for 40 min in the presence of 5 mM antipain. After incubation, the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. proLP, Precursor of the major lipoprotein; LP, major lipoprotein; SP, signal peptide.

in the cytosol or periplasm in signal peptide digestion was also suggested by the fact that no accumulation of the signal peptide was observed when TB0015 (Δ*sppA*) cells were labeled with [^{35}S]methionine (data not shown). As candidates for such proteases, Novak et al. (25) proposed oligopeptidase and protease So in the cytosol.

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