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Mutational Analysis of Plasmid R64 Thin Pilus Prepilin: the Entire Prepilin Sequence Is Required for Processing by Type IV Prepilin Peptidase

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The thin pili of IncI1 plasmid R64, which is required for conjugation in liquid media, belong to the type IV pilus family. They consist of a major subunit, the *pilS* product, and a minor component, one of the seven *pilV* products. The *pilS* product is first synthesized as a 22-kDa prepilin, processed to a 19-kDa mature pilin by the function of the *pilU* product, and then secreted outside the cell. The mature pilin is assembled to form a thin pilus with the *pilV* product. To reveal the relationship between the structure and function of the *pilS* product, 27 missense mutations, three N-terminal deletions, and two C-terminal deletions were constructed by PCR and site-directed mutagenesis. The characteristics of 32 mutant *pilS* products were analyzed. Four *pilS* mutant phenotype classes were identified. The products of 10 class I mutants were not processed by prepilin peptidase; the extracellular secretion of the products of two class II mutants was inhibited; from 11 class III mutants, thin pili with reduced activities in liquid mating were formed; from 9 class IV mutants, thin pili with mating activity similar to that of the wild-type *pilS* gene were formed. The point mutations of the class I mutants were distributed throughout the prepilin sequence, suggesting that processing of the *pilS* product requires the entire prepilin sequence.

Type IV pili are flexible, rod-like, polarly inserted surface appendages protruding from the cell surface of gram-negative bacteria including *Pseudomonas aeruginosa*, *Bacteroides nodosus*, *Neisseria gonorrhoeae*, *Moraxella bovis*, *Vibrio cholerae*, and enteropathogenic and enterotoxigenic *Escherichia coli* (9, 19, 20, 23, 27, 32). Type IV pili promote the attachment of bacterial pathogens to receptors of host cells during colonization, and they mediate the bacterial locomotion called twitching motility of *P. aeruginosa* (35) and the social gliding motility of *Myxococcus xanthus* (36). In addition, they act as receptors for pilus-specific bacteriophage (6).

Type IV pili are polymers of type IV pilin subunits (23, 27), which are produced from type IV prepilins by the function of prepilin peptidases (18). In many cases, the N-terminal amino acid of mature pilin is phenylalanine and is N-methylated. In *P. aeruginosa*, both processing of prepilin and N-methylation of mature pilin are catalyzed by a single bifunctional enzyme, the PilD protein (28). Among all type IV pilins, the N-terminal region including the cleavage site is highly conserved. Particularly, the C-terminal amino acid of the prepeptide is invariantly glycine, and the fifth amino acid of mature pilin is always glutamic acid. The C-terminal one-third of mature pilin forms a disulfide loop between two conserved cysteine residues (21, 25).

During bacterial conjugation, the donor cells harboring self-transmissible plasmids synthesize sex pili encoded by the genes on the plasmids (6). Sex pili of donor cells create a specific contact with recipient cells, leading to the formation of a mating pair. IncI1 plasmids such as R64 and ColIb-P9 form two types of sex pili, a thick rigid pilus and a thin flexible one (1, 2). Thick rigid pili are required for both surface and liquid mating, while thin flexible pili are required only for liquid mating. Cells producing R64 thin pili become sensitive to bacteriophages Iα and PR64FS, which adsorb to the shaft and tip of IncI1 thin pilus, respectively (4, 5).

DNA sequence analysis of the R64 *pil* region responsible for thin-pilus formation revealed that the *pil* region consists of 14 genes, *pilI* through *pilV*, and that several *pil* products contain amino acid sequence homology with proteins involved in type IV pilus biogenesis (11) (Fig. 1A). Thus, the R64 thin pilus was shown to belong to the type IV family, specifically group IVB, of pili.

R64 and ColIb-P9 thin pili were sedimented by ultracentrifugation from the culture medium, in which *E. coli* cells harboring R64- and ColIb-P9-derived plasmids had grown, and purified by CsCl density gradient centrifugation (13, 37). In negatively stained thin-pilus samples, long rods with a diameter of 6 nm, characteristic of type IV pili, were observed under an electron microscope. R64 and ColIb-P9 thin pili consist of a major 19-kDa pilin protein, the product of the *pilS* gene, and a minor 45-kDa protein, the product of the *pilV* gene. The amino acid sequence of the *pilS* product contains residues characteristic of a type IV prepilin, although its prepeptide is unusually long (Fig. 1B). The *pilS* product is first synthesized as a 22-kDa prepilin and then cleaved between Gly^{23} and Trp^{24} to produce a 19-kDa protein via the function of the *pilU* product, prepilin peptidase. The N-terminal amino group of the processed PilS protein appears to be modified. The C-terminal segments of the *pilV* gene are under the control of shufflon DNA rearrangement mediated by the *rci* product (15, 16). The shufflon determines the recipient specificity in liquid mating by converting seven C-terminal segments of the *pilV* product (13, 14). The *pilV* product also carries a type IV prepilin cleavage site. For-

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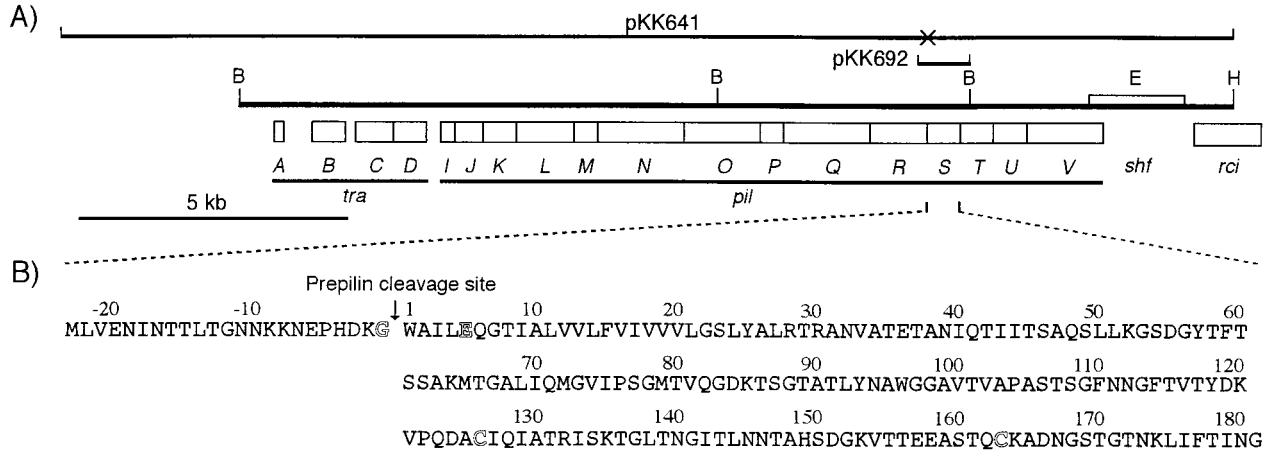

FIG. 1. (A) Organization of the *tra-pil* region of plasmid R64. The horizontal bold line represents a restriction map. B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII. The open bar above the map represents the extent of movement of the *Eco*RI site through DNA rearrangement of the shufflon. Below the map, the open reading frames are represented by open bars. *tra*, transfer; *pil*, formation of thin pilus; *shf*, shufflon; *rci*, recombinase for the shufflon. DNA regions of pKK641 and pKK692 are indicated above the map. The cross on pKK641 marks the location of the *pilS1* mutation. (B) Amino acid sequence of the PilS protein. The downward arrow indicates the type IV prepilin cleavage site. The conserved glycine, glutamic acid, and two cysteine residues are indicated by the outline letters.

mation of PilV-specific cell aggregates by ColIb-P9 and R64 thin pili was shown and suggested to play an important role in liquid mating (37).

Recently, the three-dimensional structure of the *N. gonorrhoeae* pilin was determined by X-ray crystallography (21). The monomer structure was an α-β-roll fold with an 85-Å N-terminal α-helical spine. The gross monomer structure resembles a ladle with the N-terminal half of the α-helical spine forming the handle. From the monomer structure, a model of fiber structure with a parameter of five turns per helix, 41-Å pitch, and 60-Å diameter (34) was proposed. In the model, the N-terminal α helices gather in the center of the fiber, forming a core of coiled α helices banded by a β sheet. Slight similarities including two conserved cysteine residues are noted between the amino acid sequences of R64 and *N. gonorrhoeae* pilins, suggesting that the two proteins fold similarly and then assemble to form similar fibers.

In *N. gonorrhoeae*, *P. aeruginosa*, and *V. cholerae*, amino acid substitutions were introduced into the prepeptide and highly conserved N-terminal regions of prepilin genes (3, 22, 26). The mutant genes were analyzed with respect to processing, secretion, and function. The importance of the conserved glycine in the prepeptide and some hydrophobic amino acids in the N-terminal region has been established.

This work was performed to reveal the relationship between the structure and function of the *pilS* product. Thirty-two missense and deletion mutations were introduced throughout the entire sequence of the *pilS* product by PCR and site-directed mutagenesis. The characteristics of the mutant *pilS* products were analyzed in terms of processing, secretion, and assembly to active thin pili with the *pilV* product. The activities of the thin pili composed of the mutant *pilS* genes were determined as the transfer frequency in liquid mating and the sensitivity to IncI1-specific phages.

MATERIALS AND METHODS

***E. coli* strains, plasmids, phages, and media.** *E. coli* strains, plasmids, and phages used in this study are listed in Table 1.

Luria-Bertani (LB) medium was prepared as previously described (24). The solid medium contained 1.5% agar. In the experiments using pET11a-derived plasmids, 10 μM isopropyl-β-D-thiogalactopyranoside was added to LB medium. Antibiotics were added to the liquid and solid media at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 12 μg/ml; and nalidixic acid, 20 μg/ml.

Designation of mutant *pilS* genes. Mutant *pilS* genes were designated as amino acid replacements: original amino acid-position-replaced amino acid. Since the coordinate of the N-terminal tryptophan of the mature *pilS* product was defined as +1, the initiating methionine was numbered −23. For example, replacement of the fifth glutamic acid (E) with valine (V) is denoted *pilSE5V*. The N-terminal deleted *pilS* genes were designated as the number of amino acids remaining in the prepeptide region. For example, the deletion resulting in a 10-amino-acid prepeptide is denoted as *pilSΔsp10*.

Construction of *pilS* mutants. The *pilS1* mutant of pKK641A′ was constructed by the introduction and removal of a tetracycline resistance gene cassette (10). A 22-bp DNA sequence, AATTCCCCGGATCCGGGGAATT, remaining at the *Ssp*I site and corresponding to the sixth codon of the *pilS* gene gave rise to the *pilS1* frameshift mutation.

Mutagenesis of the *pilS* gene was performed by PCR (24) using pKK692 as the template DNA. PCR amplification was performed with a PCR kit (Takara Shuzo) containing 0.1 μg of pKK692 DNA and 50 pmol each of M13 primers M4 and RV. In some experiments, 0.5 nM $MnCl_2$ was added into the reaction mixture to increase the mutation frequency. The *pilSG-1A*, *-G-1R*, *-E5V*, *-Y118F*, *-C126A*, *-C163A*, and *-K174*(Am) mutations were constructed by site-directed mutagenesis with synthetic oligonucleotides (17).

To construct *pilS* mutants carrying prepeptides shorter than the wild-type gene (*pilSΔsp10*, *-Δsp6*, and *-Δsp2*), PCR amplification was performed with synthetic oligonucleotides containing an *Nde*I site at the desired ATG initiation codon. The *Nde*I-*Bam*HI segment of the amplified DNA was inserted into the *Nde*I-*Bam*HI sites of pET11a to give pKK693 to pKK695.

Conjugal transfer and phage sensitivity. Liquid mating was performed as described previously (12). *E. coli* NF83 donor cells that harbored pKK641A′ *pilS1*, pKK661, and pKK692 with or without *pilS* mutations were grown to log phase and then mixed with an overnight culture of *E. coli* TN102 recipient cells. In the cases of pKK693 to pKK695, *E. coli* BL21 was used as the donor. The mixture was incubated for 90 min at 37°C and then plated at various dilutions onto selective media. Transfer frequency is presented as the ratio (expressed as a percentage) of the number of transconjugant to the number of donor cells.

Sensitivity of *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS* plasmids to phages Iα and PR64FS was determined as described previously (12).

Preparation of thin-pilus fraction. Crude thin-pilus fraction was prepared as described by Yoshida et al. (37), with a slight modification. *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS* plasmids were grown to optical density at 620 nm of 0.8 with shaking at 37°C. The culture medium was centrifuged three times at 9,200 × *g* for 10 min to remove cells. The supernatant was centrifuged at 140,000 × *g* for 1 h. The pellet is referred to as the thin-pilus fraction.

Western blot analysis. *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS* plasmids were recovered by centrifugation from the overnight culture (1.5 ml), resuspended in water (1 ml), and broken by sonication. Total proteins in 1.5 μl of lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell). The membrane was incubated with antipilin antiserum in Tris-buffered saline (50 mM Tris-HCl [pH 7.5], 150 mM NaCl), and the antiserum-bound proteins were then detected with a horseradish peroxidase-labeled goat anti-rabbit antiserum by using an ABC-POD kit (Wako).

TABLE 1. *E. coli* strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant genotype or phenotype	Reference or source
E. coli strains		
JM83	*ara* Δ(*lac-proAB*) *rpsL* φ80d*lacZ*Δ*M15*	33
JM109	*recA1 gyrA96 thi hsdR17 relA1 supE44* Δ(*lac-proAB*)/F′ *traD36 proAB lacI*^{q}*Z*Δ*M15*	33
CJ236	*dut-1 ung-1 thi-1 relA1*/pCJ105 (Cm^r)	17
BMH71-18	Δ(*lac-proAB*) *thi supE mutS215*::Tn*10* (Tc^r)/F′ *traD36 proAB lacI*^{q}*Z*Δ*M15*	17
NF83	*recA56 ara* Δ(*lac-proAB*) *rpsL* φ80 d*lacZ*Δ*M15*	7
TN102	W3110 Nal^r	12
BL21(DE3)	*ompT hsdS gal*/λ DE3 *lacUV5*-T7 gene 1	29
Plasmids		
pUC119	Ap^r, *lacZ′*	33
pET11a	Ap^r, T7 promoter	29
pKK641A′	Km^r, R64*drd-11* derivative carrying 18.5-kb *Bgl*II-*Hin*dIII *pil* segment and *rep* sequence	12
pKK641A′ *pilS1*	pKK641A′ carrying the *pilS1* mutation	This work
pKK661	Cm^r, pHSG576 derivative carrying 35.6-kb *Hin*dIII-*Bgl*II *tra* segment of R64*drd-11*	12
pKK692	0.8-kb *Hin*cII-*Bgl*II *pilS*$^+$ fragment in pUC119	37
Phages		
Iα		4
PR64FS		5

To analyze the *pilS* and *pilV* products in the thin-pilus fraction, aliquots of the thin-pilus fractions were subjected to SDS-PAGE followed by immunoblot detection using antipilin and anti-PilV antisera, respectively, as described above.

RESULTS

Construction of mutations in the *pilS* gene. Mutations in the *pilS* gene were constructed by the PCR method. Since *Taq* polymerase does not contain 3′-to-5′ exonuclease activity, the frequency of errors in DNA replication is high during PCR amplification using *Taq* polymerase. One amber and 21 missense mutations in the *pilS* coding sequence were obtained by PCR. In addition, one amber and six missense mutations were constructed by site-directed mutagenesis to analyze the function of specific amino acid residues in the *pilS* product. To determine the minimal prepeptide length required for processing of prepilin at the specific cleavage site, three mutant *pilS* genes (*pilS*Δ*sp10*, -Δ*sp6*, and -Δ*sp2*) encoding prepeptides shorter than that of the wild-type *pilS* gene were constructed to yield plasmids pKK693, pKK694, and pKK695, respectively. In summary, 27 missense mutations, three N-terminal deletions, and two C-terminal deletions were constructed and characterized (Table 2; see also Fig. 4).

Production and processing of prepilin molecules. To examine the activity of mutant *pilS* genes, we first constructed *pilS1* mutation by inserting a 22-bp sequence into the sixth codon of the *pilS* gene on pKK641A′ (Fig. 1A). The *pilS* product was shown to be synthesized as a 22-kDa protein, prepilin, and then processed to a 19-kDa protein by the function of the *pilU* product (37). Formation of the nonprocessed and processed products from the various *pilS* mutants was studied by immunoblot analysis using antipilin antiserum (Fig. 2; Table 2). Complementation of *pilS1* mutation by *pilS*$^+$ plasmid pKK692 was demonstrated, since the 22- and 19-kDa proteins were detected as the wild-type *pilS* gene products in *E. coli* cells harboring pKK641A′ *pilS1* and pKK692, while neither protein was detected in cells harboring pKK641A′ *pilS1* (Fig. 2, lanes 1 and 2). Only the 22-kDa protein was detected in cells harboring pKK692 (lane 3). Less than half of the *pilS* product from the multicopy *pilS* gene was processed by prepilin peptidase in *E. coli* cells harboring pKK641A′ *pilS1* and pKK692, while most of the prepilin was processed in cells harboring pKK641A′ (data not shown). In both cases, approximately the same amount of mature pilin was detected from the same amount of cells. These results indicate that the surplus prepilin produced from the multicopy *pilS* gene was not processed, suggesting that the *pilU* product is saturated.

The products of the mutant *pilS* genes in *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS* plasmids were detected by immunoblot analysis (Fig. 2). Note that extracts from similar numbers of *E. coli* cells were loaded in each lane of Fig. 2. Similar amounts of the PilS-related proteins (prepilin and mature pilin) were observed from all *pilS* mutants except *pilSV16D* and two amber mutants, while the amounts of the products from *pilSV16D* and two amber mutants were less than those from the wild-type *pilS* gene or the other *pilS* mutants. The electrophoretic mobilities of prepilins in SDS-PAGE from all *pilS* mutants were similar to that of the wild-type prepilin, with the exception of three N-terminal deletions, *pilSV16D*, and two amber mutants. The mobilities of the products from the three N-terminal deletion mutants and *pilSQ71*(Am) mutant were faster than that of wild-type prepilin, as expected, while the mobilities of the products from *pilSV16D* and *K174* (Am) mutants were slower than that of the wild-type prepilin.

Among six missense mutants in the prepeptide region, both the 22- and 19-kDa proteins were produced from the four mutants (*pilSE-20Q*, *-N-7D*, *-N-7K*, and *-K-2R*), indicating that their products were processed normally (Fig. 2). In contrast, the products of the *pilSG-1A* and *-G-1R* mutants could not be processed at all. Among three N-terminal deletion mutants, the 10-amino-acid-residue prepeptide was cleaved off normally from the *pilS*Δ*sp10* product. The six-amino-acid-residue prepeptide was also cleaved off from the *pilS*Δ*sp6* product but at a reduced rate. The processing of the *pilS*Δ*sp2* product was obscure, since the sizes of the precursor and processed forms from this mutant were too close to be separated by SDS-PAGE. Among 21 missense mutations in the mature pilin region, the products of five mutants (*pilSV16D*, *-A47P*, *-Y118N*, *-C163A*, and *-L175P*) could not be processed, while those of the other mutants were processed normally (Fig. 2). Neither of the products of two amber mutants was processed. It is noteworthy that in the three mutants [*pilSV16D*, *-Q71*(Am), and *-K174*(Am)] producing nonprocessed products, the levels of

TABLE 2. Characteristics of the products of various mutant *pilS* genes

Plasmid	Whole-cell extract[a]		Thin-pilus fraction[b]		Transfer frequency[c]		Sensitivity to phages[d]	
	Prepilin	Processing	PilS	PilV	*pilS* activity	Transdominance	PR64FS	Iα
pKK692	+	+	+	+	1.2 ± 0.4	1.4 ± 0.4	s	s
None	−				<0.0001	1.2	r	r
pKK693 (*pilSΔsp10*)	+	+	+	NT	1.2	NT	s	s
pKK694 (*pilSΔsp6*)	+	+	±	NT	0.4	NT	s	s
pKK695 (*pilSΔsp2*)	+	?	−	NT	<0.0001	NT	r	r
pKK692 *pilSE-20Q*	+	+	+	+	1.1	1.1	s	s
pKK692 *pilSN-7D*	+	+	+	+	1.4	1.6	s	s
pKK692 *pilSN-7K*	+	+	+	+	0.85	1.2	s	s
pKK692 *pilSK-2R*	+	+	+	+	0.81	1.25	s	s
pKK692 *pilSG-1A*	+	−	−	−	<0.0001	0.43	r	r
pKK692 *pilSG-1R*	+	−	−	−	<0.0001	0.66	r	r
pKK692 *pilSE5V*	+	+	−	−	<0.0001	0.76	r	r
pKK692 *pilSG7E*	+	+	+	+	0.0046	0.97	s	r
pKK692 *pilSI9L*	+	+	+	+	0.062	1.4	s	ps
pKK692 *pilSL14F*	+	+	+	+	0.062	1.5	s	ps
pKK692 *pilSV16D*	±	−	−	−	<0.0001	0.05	r	r
pKK692 *pilSL21Q*	+	+	+	+	0.13	0.68	s	r
pKK692 *pilSA47P*	+	−	−	−	<0.0001	0.18	r	r
pKK692 *pilSL51Q*	+	+	+	+	0.03	1.1	s	s
pKK692 *pilSK64R*	+	+	+	+	1.6	1.6	s	s
pKK692 *pilSM65T*	+	+	+	+	1.6	1.7	s	s
pKK692 *pilSQ71*(Am)	±	−	−	−	<0.0001	0.38	r	r
pKK692 *pilSN112S*	+	+	+	+	0.29	1.2	s	s
pKK692 *pilSY118N*	+	−	−	−	<0.0001	0.12	r	r
pKK692 *pilSY118F*	+	+	+	+	1.1	1.1	s	s
pKK692 *pilSC126A*	+	+	−	−	<0.0001	0.09	r	r
pKK692 *pilSI127F*	+	+	+	+	0.32	0.98	s	s
pKK692 *pilST147S*	+	+	+	+	0.15	1.2	s	s
pKK692 *pilSE157D*	+	+	+	+	0.39	1.25	s	s
pKK692 *pilSC163A*	+	−	−	−	<0.0001	0.17	r	r
pKK692 *pilSS169G*	+	+	+	+	0.29	1.1	s	s
pKK692 *pilSK174*(Am)	±	−	−	−	<0.0001	0.08	r	r
pKK692 *pilSL175P*	+	−	−	−	<0.0001	0.37	r	r
pKK692 *pilSI179T*	+	+	+	+	1.6	1.7	s	s

[a] Presence of 22-kDa (prepilin) and 19-kDa (processing) proteins reacted with antipilin antiserum in whole-cell extract; summarized from data in Fig. 2.
[b] Presence of 19-kDa (PilS) and 45-kDa (PilV) proteins reacted with antipilin and anti-PilVA′ antisera, respectively, in thin-pilus fraction; summarized from data in Fig. 3. +, detected; −, not detected; ±, small amount detected; NT, not tested.
[c] Ratio (expressed as percentage) of number of transconjugants to number of donor cells. *E. coli* NF83 donor cells carried pKK661, pKK641A′ *pilS1*, and the indicated plasmid (*pilS* activity) or pKK661, pKK641A′ and the indicated plasmid (transdominance).
[d] s, sensitive; r, resistant; ps, partially sensitive.

expression of the products were also suppressed as described above. Formation of nonprocessed products from the mutants located in the mature pilin region is of particular interest, since it indicates the importance of mature pilin region amino acids for processing of prepilin.

Formation of thin pilus from the mutant *pilS* genes. The *pilS* product may be processed to lose the prepeptide, be translocated across the cell membrane, and polymerize to form thin pili. R64 thin pili detached from *E. coli* cells harboring pKK641A′ could be recovered by ultracentrifugation from the culture medium in which the cells had grown (thin-pilus fraction) (37). The 19-kDa protein, mature pilin, was detected in the thin-pilus fraction from the culture media of *E. coli* cells harboring pKK641A′ *pilS1* and pKK692 by immunoblot analysis using antipilin antiserum, while the 22-kDa prepilin was not (Fig. 3A, lane 1). The *pilS* product was not detected in the thin-pilus fraction from cells harboring pKK641A′ *pilS1* or those with pKK692 (lanes 2 and 3).

The thin-pilus fractions from *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS* plasmids were subjected to immunoblot analysis. Among the *pilS* mutants whose products were processed normally, all except *pilSΔsp6*, *pilSE5V*, and *pilSC126A* produced thin pili outside the cells at levels similar to that of the wild-type gene (Fig. 3A; Table 2). The *pilSΔsp6* mutant produced thin pili outside the cells at a lower level. Thin pili were not detected from the culture media of the *pilSE5V* and *C126A* mutants. The results of whole-cell enzyme-linked immunosorbent assay analysis paralleled that of immunoblot analysis (data not shown). Electron microscopic observation indicated that thin-pilus fractions from cells with mutant *pilS* contained fibrous structures similar to those from cells with wild-type *pilS* (data not shown). In the *pilSΔsp2* and *pilSG-1A*, *-G-1R*, *-V16D*, *-A47P*, *-Q71*(Am), *-Y118N*, *-C163A*, *-K174*(Am), and *-L175P* mutants, for which mutant prepilins were not processed, no thin pili were detectable in the culture media (Fig. 3A).

R64 thin pili were shown to contain a minor subunit, the *pilVA′* product (37). The *pilVA′* product was detected in the thin-pilus fraction prepared from the culture media of *E. coli* cells harboring pKK641A′ *pilS1* and pKK692 as a 45-kDa protein by immunoblot analysis using anti-PilVA′ antiserum, while the *pilVA′* product was not detected in the same fraction from cells harboring pKK641A′ *pilS1* or those harboring pKK692 (Fig. 3B). The thin-pilus fractions from the culture media of *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS*

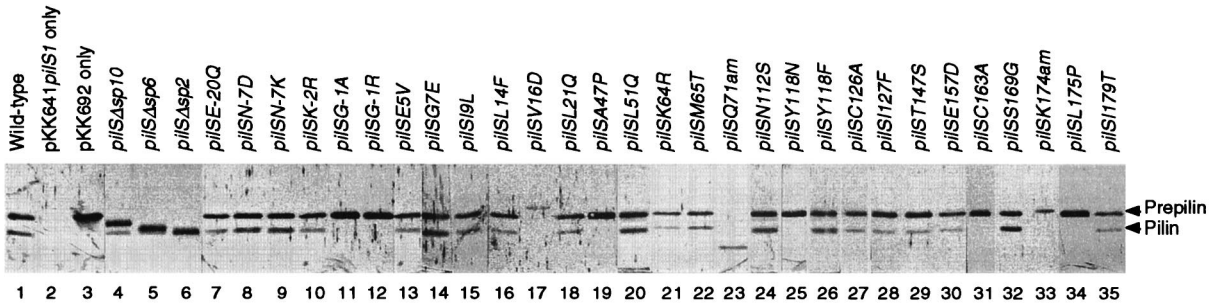

FIG. 2. Expression and processing of the products of various *pilS* mutants. Whole extracts of *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS* plasmids were separated by SDS-PAGE and subjected to Western blot analysis using antipilin antiserum. Lanes: 1, pKK692 (wild-type *pilS*); 2, without *pilS* plasmid; 3, without pKK641A′ *pilS1*; 4 to 6, pKK693 to pKK695; 7 to 35, pKK692 with the indicated *pilS* mutations. The positions of prepilin (22 kDa) and pilin (19 kDa) are indicated on the right.

plasmids were subjected to immunoblot analysis. The thin pili from all of the piliated mutants (see above) contained the *pilVA′* product; the *pilSΔsp10* and *pilSΔsp6* mutants were not analyzed (Fig. 3B; Table 2).

Activity of the mutant *pilS* genes in R64 transfer. To determine the activity of the mutant *pilS* genes during conjugation, the pKK641-pKK661 system was used (12). In this system, since a lack of the *rci* gene prevents DNA rearrangement of the shufflon, the transfer frequency of R64 in liquid mating can be accurately estimated. *E. coli* donor cells harboring pKK641 and pKK661 transmitted pKK661 carrying the *oriT* sequence into the recipient cells by conjugation. The activity of the mutant *pilS* genes was determined by measuring the effects of the addition of the mutant *pilS* plasmids on the transfer frequency in liquid mating from donor cells harboring pKK641A′ *pilS1* and pKK661. The transfer frequency from donor cells harboring pKK641A′ *pilS1* and pKK661 was less than 0.0001% (Table 2). When pKK692 carrying the wild-type *pilS* gene was introduced into donor cells, the transfer frequency was increased to 1.2%.

Different levels of recovery in the transfer frequency were observed by introducing various mutant *pilS* genes into donor cells harboring pKK641A′ *pilS1* and pKK661 (Table 2). Among three N-terminal deletion mutants, donor cells harboring the *pilSΔsp10* mutant exhibited a transfer frequency similar to that of the wild-type gene during liquid mating. For the *pilSΔsp6* mutant, which formed thin pili at a lower level, the transfer frequency was 10-fold lower than that of the wild-type gene. The *pilSΔsp2* mutant did not exhibit *pilS* activity.

For nine missense mutants (*pilSG-1A*, *-G-1R*, *-E5V*, *-V16D*, *-A47P*, *-Y118N*, *-C126A*, *-C163A*, and *-L175P*) as well as two nonsense mutants [*pilSQ71*(Am) and *K174*(Am)], the products of which were not processed or secreted outside the cells, no transfer was observed, as expected (Table 2). For eight piliated mutants (*pilSE-20Q*, *-N-7D*, *-N-7K*, *-K-2R*, *-K64R*, *-M65T*, *-Y118F*, and *-I179T*), transfer frequencies similar to that of the wild-type gene were observed. For 10 piliated mutants (*pilSG7E*, *-I9L*, *-L14F*, *-L21Q*, *-L51Q*, *-N112S*, *-I127F*, *-T147S*, *-E157D*, and *-S169G*), transfer frequencies were lower than that of the wild-type *pilS* gene. The transfer frequencies of these mutants varied from allele to allele. In particular, the transfer frequency of the *pilSG7E* mutant was 100-fold lower than that of the wild-type gene.

Transdominance of the mutant *pilS* genes over the wild-type *pilS* gene. Addition of pKK692 (multicopy wild-type *pilS*) to donor cells harboring pKK641A′ and pKK661 had no effect on the transfer frequency in liquid media (Table 2). The effects of introducing multiple copies of mutant *pilS* genes to donor cells harboring pKK641A′ and pKK661 on the transfer frequency were studied to test whether the *pilS* mutants were dominant negative over the wild-type *pilS* gene.

The introduction of 12 mutants [*pilSG-1A*, *-G-1R*, *-E5V*, *-V16D*, *-L21Q*, *-A47P*, *-Q71*(Am), *-Y118N*, *-C126A*, *-C163A*,

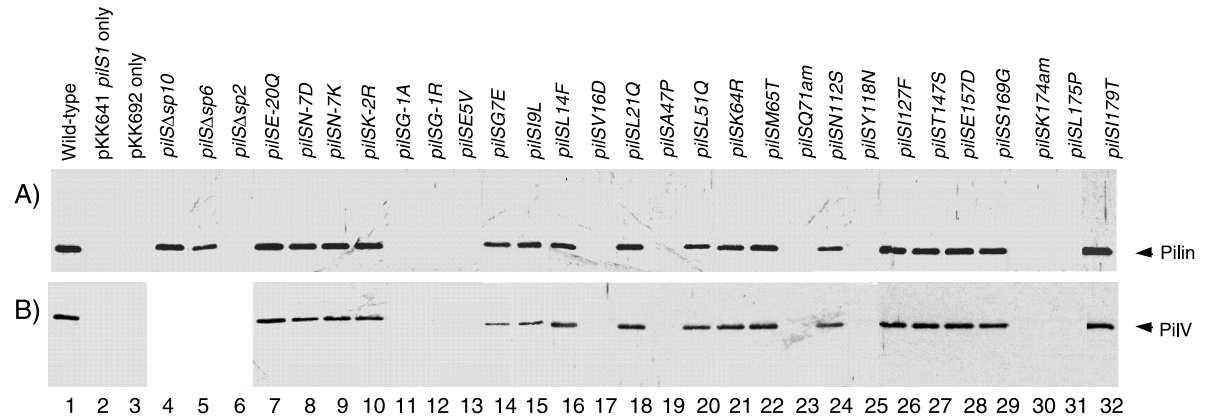

FIG. 3. Formation of thin pili with PilVA′ protein from various *pilS* mutants. Thin-pilus fractions were prepared from the culture media of *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS* plasmids. Proteins were separated by SDS-PAGE and subjected to Western blot analysis using antipilin antiserum (A) and anti-PilVA′ antiserum (B). Lanes: 1, pKK692 (wild-type *pilS*); 2, without *pilS* plasmid; 3, without pKK641A′ *pilS1*; 4 to 6, pKK693 to pKK695; 7 to 32, pKK692 with the indicated *pilS* mutations. The positions of pilin (19 kDa) and PilVA′ protein (45 kDa) are indicated on the right.

-K174am(Am), and *-L175P*] into donor cells harboring pKK641A′ and pKK661 decreased the transfer frequencies, indicating the transdominant character of these mutants. All of these mutants except *pilSL21Q* lacked *pilS* activity during liquid mating.

Sensitivity to phages Iα and PR64FS. Upon infection of bacterial cells harboring IncI1 plasmids, phages Iα and PR64FS specifically adsorb to the shaft and tip of the thin pili formed by IncI1 plasmids, respectively, and subsequently the infected cells produce progeny phage (4, 5). Therefore, the sensitivity of cells to phages Iα and PR64FS can be used as the indication of thin-pilus formation. Although *E. coli* cells harboring pKK641A′ *pilS1* were resistant to phages Iα and PR64FS, cells harboring pKK641A′ *pilS1* and pKK692 were sensitive to them (Table 2).

The sensitivities to these phages of *E. coli* cells harboring pKK641A′ *pilS1* and the mutant *pilS* plasmids were determined. Cells harboring 16 mutant *pilS* genes (*pilSΔsp10*, *-Δsp6*, *-E-20Q*, *-N-7D*, *-N-7K*, *-K-2R*, *-L51Q*, *-K64R*, *-M65T*, *-N112S*, *-Y118F*, *-I127F*, *-T147S*, *-E157D*, *-S169G*, and *-I179T*) in addition to pKK641A′ *pilS1* exhibited Iα and PR64FS phage sensitivity similar to that of cells with the wild-type *pilS* gene (Table 2). Cells with 12 mutant genes [*pilSΔsp2*, *-G-1A*, *-G-1R*, *-E5V*, *-V16D*, *-A47P*, *-Q71*(Am), *-Y118N*, *-C126A*, *-C163A*, *-K174* (Am), and *-L175P*], which exhibited no *pilS* activity in liquid mating, were resistant to both phages. Cells with two mutations (*pilSI9L* and *-L14F*) were sensitive to PR64FS but partially sensitive to Iα. Cells with two mutations (*pilSG7E* and *-L21Q*) were sensitive to PR64FS but resistant to Iα. These four mutations were located within the N-terminal hydrophobic region of mature pilin.

DISCUSSION

Genetic analysis was performed to reveal the relationship between the structure and function of the R64 *pilS* product. Thirty-two *pilS* mutants were characterized with respect to the processing, extracellular secretion, and assembly to active thin pilus (Table 2). The activities of thin pili produced from these *pilS* mutants were analyzed as the transfer frequency in liquid media and the sensitivity to phages Iα and PR64FS. From these results, the *pilS* mutants can be classified into four classes (Fig. 4). The products of 10 mutants [*pilSΔsp2*, *-G-1A*, *-G-1R*, *-V16D*, *-A47P*, *-Q71*(Am), *-Y118N*, *-C163A*, *-K174*(Am), and *-L175P*] were not processed by prepilin peptidase (class I). The extracellular secretion of the products of two mutants (*pilSE5V* and *-C126A*) was inhibited, although these products were processed at the normal rate (class II). From 11 mutants (*pilSΔsp6*, *-G7E*, *-I9L*, *-L14F*, *-L21Q*, *-L51Q*, *-N112S*, *-I127F*, *-T147S*, *-E157D*, and *-S169G*), thin pili with reduced activities during liquid mating were formed (class III). From nine mutants (*pilSΔsp10*, *-E-20Q*, *-N-7D*, *-N-7K*, *-K-2R*, *-K64R*, *-M65T*, *-Y118F*, and *-I179T*), thin pili with activities similar to that of the wild-type *pilS* gene were formed (class IV).

Prepeptide region. The products of four mutants in the prepeptide region (*pilSE-20Q*, *-N-7D*, *-N-7K*, and *-K-2R*) were processed as efficiently as that of the wild-type strain and used in the formation of normal thin pili. Since these mutants are expected to produce normal pilin after processing, it is reasonable that thin pili from these mutants exhibited normal transfer frequency. A series of mutations were introduced into the prepeptide sequence of the *pilA* gene in *P. aeruginosa* (26). Normal pili were produced from all single or double *pilA* mutants except Gly^{-1}, in fair agreement with the results of the R64 *pilS* mutants.

The products of the R64 *pilSG-1A* and *pilSG-1R* mutants could not be processed. All type IV prepilins have a glycine

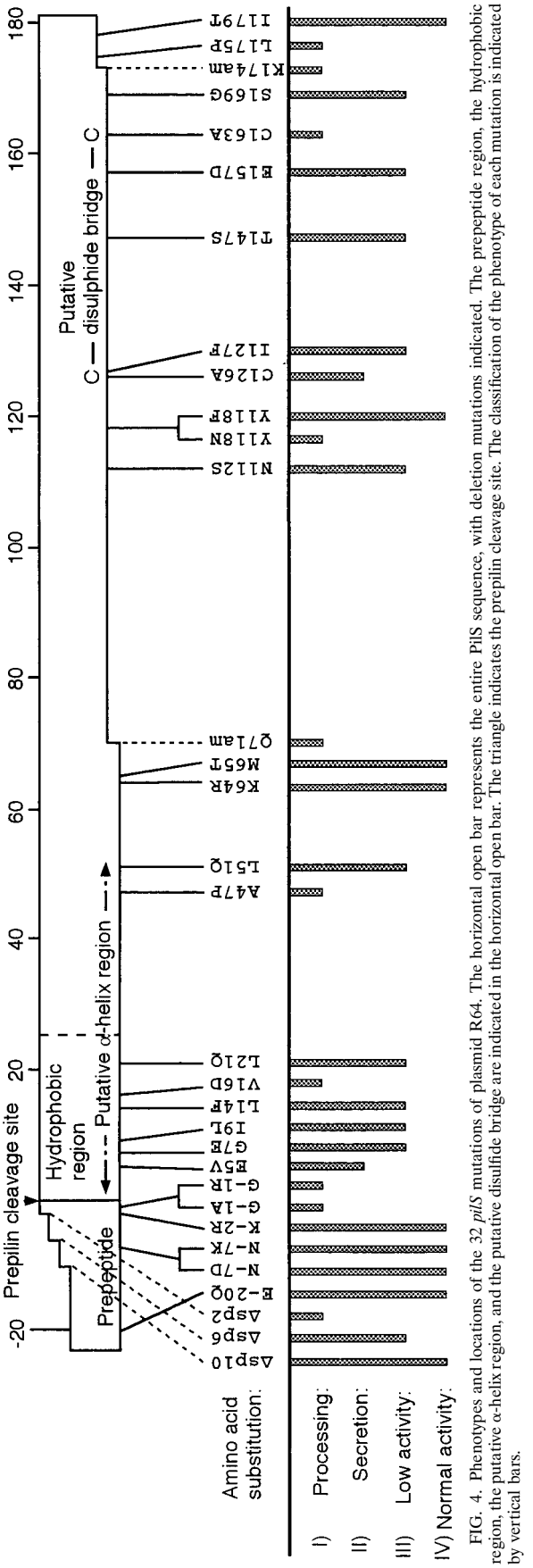

FIG. 4. Phenotypes and locations of the 32 *pilS* mutations of plasmid R64. The horizontal open bar represents the entire PilS sequence, with deletion mutations indicated. The prepeptide region, the hydrophobic region, the putative α-helix region, and the putative disulfide bridge are indicated in the horizontal open bar. The triangle indicates the prepilin cleavage site. The classification of the phenotype of each mutation is indicated by vertical bars.

residue at the C terminus of the prepeptide. In *P. aeruginosa pilA* and *V. cholerae tcpA*, the products of all Gly^{-1} mutants except *pilAG-1A* could not be processed, while the *pilAG-1A* product was partially processed (3, 26). In contrast, in the R64 system, even the *pilSG-1A* product could not be processed.

The prepeptides of typical type IV prepilins consist of 6 to 7 amino acids (23, 27), while R64 *pilS* product has a longer prepeptide consisting of 23 amino acids. The product of the *pilSΔsp10* mutant carrying a 10-amino-acid prepeptide was processed normally, indicating that the entire sequence of prepeptide is not required for the processing of the R64 *pilS* product. The product of the *pilSΔsp6* mutant carrying a six-amino-acid prepeptide was processed partially and produced thin pili at a reduced level. This mutant is the only class III mutant which exhibits a quantitative reduction in thin-pilus production. The product of the *pilSΔsp2* mutant carrying only glycine most likely is not processed, as was found for *P. aeruginosa pilA* (26).

Putative α-helix region. By analogy with *N. gonorrhoeae* pilin (21), approximately 53 N-terminal amino acids of R64 pilin are predicted to form an α-helical spine with the N-terminal half protruding from a C-terminal globular head. Among eight mutations located within this region, the *pilSV16D* and *-A47P* were defective in processing. The *pilSE5V* product was susceptible to processing but could not be secreted extracellularly. Similar phenotypes of the *E5K* and *E5V* mutations were reported for *N. gonorrhoeae pilE* and *P. aeruginosa pilA* genes (22, 26). The remaining five mutant genes (*pilSG7E*, *-I9L*, *-L14F*, *-L21Q*, and *-L51Q*) formed extracellular thin pili with the PilVA′ protein. However, thin pili from these mutants exhibited a transfer frequency lower than that of the wild-type *pilS* gene in liquid mating (Table 2). These results suggest an important role of the putative α-helix region in the function of R64 thin pili. In fiber formation, the N-terminal hydrophobic regions of pilin are predicted to assemble with one another to form coiled α helices (21). It is noteworthy that even an isoleucine-to-leucine replacement was not tolerated in this region. No class IV mutants were obtained within this region.

C-terminal region. As was found for *N. gonorrhoeae* pilin (21), the C-terminal two-thirds of R64 pilin is predicted to fold into a globular structure. The products of two amber mutant genes [*pilSQ71*(Am) and *pilSK174*(Am)] could not be processed. The *pilSK174*(Am) mutant lacks only seven C-terminal amino acids. The inability to be processed in *pilSK174*(Am) and *pilSL175P* indicates the importance of the C-terminal segment of the *pilS* product in processing. In addition, two class I mutants (*pilSY118N* and *pilSC163A*) are present in this region. Although the *pilSY118N* mutant was a class I mutant, the product of the *pilSY118F* mutant produced active thin pili, suggesting that Tyr^{118} is important for the formation of a hydrophobic core in the pilin molecule as in *N. gonorrhoeae* pilin (21).

Various type IV pilins are thought to be stabilized by a disulfide bridge between two conserved cysteine residues (21, 25). In R64 pilin, Cys^{126} and Cys^{163} are predicted to form a disulfide bridge (Fig. 1B). The *pilSC126A* and *pilSC163A* mutations disrupting the disulfide bridge between the two cysteine residues were classified as class II and I mutants, respectively, signifying the important role of the disulfide bridge in protein folding of the R64 *pilS* product. In contrast to our results, in bundle-forming pili of enteropathogenic *E. coli*, two cysteine-to-serine mutations as well as introduction of *dsbA* mutation resulted in reduced levels of pilin (38). The reason for the apparent discrepancy is not known.

Characteristics of the four mutant classes. The class I and II mutants exhibited nonpiliated phenotype. To our surprise, the mutation points of the class I mutants were distributed throughout the prepilin sequence. The presence of class I mutants in the *pilS* C-terminal region indicates that the entire prepilin must be made prior to cleavage of the prepeptide. These results exhibit a striking contrast to the case of the general secretory pathway, for which the importance of the signal peptide has been established (23). By using the signal peptide of the *E. coli ompA* gene, a plasmid vector for the secretion of foreign protein was constructed (8). In the case of type IV prepilin, replacements of many amino acids in the prepeptide sequence, but not of Gly^{-1}, were permissible (reference 26 and this study). Many class I and II mutations appear to disrupt PilS protein folding by the introduction of deletions, the introduction of proline, or the removal of the disulfide bridge. It is possible that the folded PilS protein structure is required for cleavage of its prepeptide.

In *N. gonorrhoeae* pilus-positive (P^+)-to-P^- phase transitions, a P^- rp^+ variant (P^-, but can be reverted to P^+) of *pilE* was characterized (30, 31). In the P^- rp^+ cells, type IV pilus was not produced, while a mutant protein which reacted with antipilin antibody was produced and processed. Hence, the P^- rp^+ phenotype is very similar to that of the R64 class II *pilS* mutants. The difference in the amino acid sequence between the P^- rp^+ strain and its P^+ revertant was 9 amino acid replacements within a 12-amino-acid segment of the C-terminal hypervariable region of the *pilE* gene. These results suggest that the formation of class II (and most likely class I) mutations by antigenic variation in the *N. gonorrhoeae pilE* gene results in P^+-to-P^- phase transitions.

The class I and II mutants exhibited a transdominant phenotype over the wild-type allele (Table 2), suggesting that overproduced mutant proteins may competitively inhibit some step(s) in thin-pilus formation from the wild-type PilS protein.

The products of the class III mutants formed extracellular thin pili with a low transfer frequency during liquid mating. Among 11 class III mutants, all except *pilSΔsp6* produced the altered pilin with various degrees of *pilS* activity, while the *pilSΔsp6* mutant exhibited a quantitative mutant phenotype. *E. coli* cells harboring the class III mutants were sensitive to phage PR64FS, but their sensitivity to Iα was allele dependent, exhibiting a sensitive, partially sensitive, or resistant phenotype. Most mutants exhibiting resistance or partial sensitivity to Iα clustered within the N-terminal hydrophobic region.

Why do the class III mutants exhibit the low-frequency phenotype during liquid mating? At the beginning of liquid mating, the R64 thin pili in donor cells are predicted to attach to the surface of the recipient cells, resulting in the formation of donor-recipient cell aggregates (13, 14, 37). At that time, one of seven PilV proteins selected by the shufflon DNA rearrangement recognizes the surface receptor of the recipient cells and determines the recipient specificity. Subsequent steps promoted by thin and thick pili and other transfer genes eventually transfer R64 DNA into the recipient cells.

One possible reason for the low-frequency phenotype is that the mutant pilin forms a weak thin pilus, which is broken at a rate higher than that of the wild-type pilin. The weak thin pilus is expected to cause a defect in donor-recipient complex formation. Another possible reason for the low-frequency phenotype is related to the retraction model of pilus (6). Various pili are thought to outgrow and retract. Retraction may be important in establishing the donor-recipient complex in conjugation and phage infection. It is possible that the R64 thin pilus consisting of mutant pilin is defective in the retraction process.

It was reported that in the *V. cholerae* Tcp system, type IV pili were formed by two *tcpA* mutants (V9M and V20T) but were inactive in autoagglutination and colonization (3). The R64 thin-pilus system seems to be one of the best for such

analyses, since it allows for quantitative estimation of the function of type IV pilus.

The isolation of four classes of *pilS* mutations leads us to propose the following model for the process of R64 thin-pilus formation: (i) the R64 *pilS* product is synthesized, (ii) the R64 *pilS* product is processed by prepilin peptidase to yield R64 pilin, and (iii) R64 pilin is secreted across the cell membrane and assembled with the PilV protein.

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