Genetic Mapping and Properties of Filamentous Mutations in *Bacillus subtilis*

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Seven filamentous (*fil*) mutants were isolated from *B. subtilis*, and the mutations were mapped by means of lysed-protoplast transformation. Five of the mutations were linked to *aroD* and the others to *pyrD*. *rgn* mutations, which lead to a decrease in autolysin(s) and the formation of filaments, were also linked to *aroD*, and the mapping order was *rgn-dnaE-aroD*. On comparison with other reported filamentous mutations (*lyt-1*, *lyt-2* and *lyt-152*), *fil-1*, *fil-3* to *-6*, *rgn* and the above *lyt* mutations were determined to be in the same locus. All of the seven *fil* strains lacked flagella and showed decreased autolysin activity. Among them, only mutants having *aroD*-linked mutations showed low competency. Protease assay results indicated that *rgn* mutants produce a several times higher amount of the enzyme than the parent strain, and the initiation time for the production in *rgn* mutants was two hours earlier than in the parent strain.

Bacillus subtilis is one of the most extensively genetically and biochemically characterized gram-positive bacteria, and it exhibits peculiar features (e.g., extracellular enzyme production and sporulation) that *Escherichia coli* does not.¹⁾ But, fundamental research on the cell wall and membrane of *B. subtilis* lags far behind that in the case of *E. coli*,²⁾ and also the secretion mechanism remains obscure.

Being exceptional, several pleiotropic mutations (*pap*, *amyB* and *sacU*^h) that lead to simultaneous increases in the production of α -amylase, serine and metal proteases, and levansucrase are known.^{3~5)} The mutations, mapped in the *sacU* locus, lead to a lack of both competency and flagella, and a decreased amount of autolysin(s). On the other hand, *rgn* mutants, originally isolated to increase the regeneration of protoplasts, had some similar properties to *sacU* (e.g., a low autolysin amount, non-competency and a lack of flagella).⁶⁾

Grant and Simon investigated flagella-minus (*fla*) mutants,⁷⁾ and later Fein and Rogers also investigated flagella-minus mutants which exhibit a decreased amount of autolysin(s).⁸⁾ Recently, Pooley and Karamata⁹⁾ reported the mapping of autolysin mutations (*lyt*), which showed that *lyt-1* and *lyt-2* are located in *flaD* (219°) and *lyt-15* in *flaA* (145°). The other *fla* loci are *flaB* (149°) and *flaC* (315°). We also investigated the properties and loci of *lyt* mutants and obtained the following results: (1) a *lyt* mutant, Ni15, has three autolysin-deficient (*lyt*) mutations (*lyt-15*, *lyt-151* and *lyt-152*), (2) *lyt-15* is linked to *pyrD1* (135°), *lyt-151* to *hisA* (299°) and *lyt-152* to *aroD* (230°), (3) these mutations lead to a lack of flagella, and (4) *lyt-151* and *lyt-152* mutants are filamentous whereas the *lyt-15* mutant is rod-shaped (Mol. Gen. Genet., *in press*).

In this report, we describe the isolation of filamentous (*fil*) mutants and the comparison of genetic loci and biochemical properties be-

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TABLE I. *Bacillus subtilis* STRAINS

Strain designation	Genotype	Reference, source or derivation
168S	<i>strA trpC2</i>	T. Seki
YS11	<i>purB6 arg-15 leuB8</i>	T. Seki
AG5	<i>purB6 arg-15 rgn-1 leuB8</i>	Akamatsu and Sekiguchi (1983)
AH6	<i>purB6 arg-15 rgn-2 leuB8</i>	Akamatsu and Sekiguchi (1983)
1A22	<i>ilvA1 metB5 dnaE20</i>	BGSC ^a
1A483	<i>metC3 lyt-1</i>	BGSC; FJ3 strain
1A484	<i>metC3 lyt-2</i>	BGSC; FJ6 strain
1A504	<i>xin-15 lyt-15 thyA thyB trpC2 lyt-151 lyt-152</i>	BGSC; Ni15 strain; * ^b
CT11-6	<i>hisH2 rgn-1</i>	*
AC310	<i>purB tet-1^c</i>	*
AC325	<i>purB rgn-1</i>	Tfm (AC310: CT11-6 DNA, Tc ^r Rgn ⁻) ^d
AC327	<i>purB his-1</i>	*
AC343	<i>strA met-1 glt-1 trpC2 leu-2 his-1 purA</i>	*
AC408	<i>pyrD his-1</i>	*
AC415	<i>lys aroD120 asaA4 leu-2</i>	*
AC416	<i>trpC2 lys leu-2</i>	Akamatsu and Sekiguchi (1987)
AC551	<i>purB his-1 fil-1</i>	NTG mutagenesis of AC327, Fil ⁻ ^e
AC552	<i>purB his-1 fil-2</i>	NTG mutagenesis of AC327, Fil ⁻
AC553	<i>purB his-1 fil-3</i>	NTG mutagenesis of AC327, Fil ⁻
AC554	<i>purB his-1 fil-4 glu-1 com-1</i>	NTG mutagenesis of AC327, Fil ⁻ Glu ⁻ Com ^{-f}
AC555	<i>purB his-1 fil-5</i>	NTG mutagenesis of AC327, Fil ⁻
AC556	<i>purB his-1 fil-6</i>	NTG mutagenesis of AC327, Fil ⁻
AC557	<i>purB his-1 fil-7</i>	NTG mutagenesis of AC327, Fil ⁻
AC601	<i>lys fil-1 leu-2</i>	Tfm (AC415: AC551 DNA, Aro ⁺ Asa ⁺ Fil ⁻)
AC602	<i>purB fil-2</i>	Tfm (AC310: AC552 DNA, Tet ⁺ Fil ⁻)
AC603	<i>lys fil-3 leu-2</i>	Tfm (AC415: AC553 DNA, Aro ⁺ Asa ⁺ Fil ⁻)
AC604	<i>lys fil-4 leu-2 com-1</i>	Tfm (AC415: AC554 DNA, Aro ⁺ Asa ⁺ Fil ⁻ Com ⁻)
AC605	<i>lys fil-5 leu-2</i>	Tfm (AC415: AC555 DNA, Aro ⁺ Asa ⁺ Fil ⁻)
AC606	<i>lys fil-6 leu-2</i>	Tfm (AC415: AC556 DNA, Aro ⁺ Asa ⁺ Fil ⁻)
AC607	<i>purB fil-7</i>	Tfm (AC310: AC557 DNA, Tet ⁺ Fil ⁻)
AC609	<i>his-1 fil-2</i>	Tfm (AC408: AC552 DNA, Ura ⁺ Fil ⁻)
AC610	<i>his-1 fil-7</i>	Tfm (AC408: AC557 DNA, Ura ⁺ Fil ⁻)
AC620	<i>lys rgn-2 leu-2</i>	Tfm (AC415: AH6 DNA, Aro ⁺ Asa ⁺ Rgn ⁻)
AC621	<i>lys lyt-1 leu-2</i>	Tfm (AC415: 1A483 DNA, Aro ⁺ Asa ⁺ Lyt ⁻)
AC622	<i>lys lyt-2 leu-2</i>	Tfm (AC415: 1A484 DNA, Aro ⁺ Asa ⁺ Lyt ⁻)
AC623	<i>lys lyt-152 leu-2</i>	Tfm (AC415: 1A504 DNA, Aro ⁺ Asa ⁺ Lyt ⁻)
AC703	<i>lys rgn-1 leu-2</i>	Tfm (AC415: AG5 DNA, Aro ⁺ Asa ⁺ Rgn ⁻)
AC705	<i>lys dnaE20 aroD120 asaA4</i>	Tfm (AC415: 1A22 DNA, Leu ⁺ Ts ^g)
AC708	<i>lys rgn-1 dnaE20</i>	Tfm (AC703: AC705 DNA, Leu ⁺ Ts)
AC790	<i>lys fil-4 leu-2</i>	Tfm (AC415: AC604 DNA, Aro ⁺ Asa ⁺ Fil ⁻)

^a The *Bacillus* Genetic Stock Center.^b Akamatsu and Sekiguchi *Mol. Gen. Genet.*, in press.^c Tetracycline-sensitive mutation.^d Tfm, transformation; Tfm (AC310: CT11-6 DNA, Tc^r Rgn⁻) denotes the Tc^r Rgn⁻ transformant of AC310 with CT11-6 DNA.^e Filamentous phenotype.^f Glu⁻ and Com⁻ denote the glutamic acid requirement and lower competency phenotypes, respectively. These mutations in addition to *fil* occurred in AC554 on NTG mutagenesis. The details of Com⁻ are given in the text.^g Temperature-sensitive.

tween the previously reported mutations and *fil* ones. We also describe the mapping of *rgn* mutations by means of protoplast fusion and lysed-protoplast transformation.

MATERIALS AND METHODS

Media. For bacterial growth, nutrient broth containing 10 g of beef extract (Difco), 10 g of bacto-peptone (Difco) and 2 g of NaCl per liter (pH 7.0) was used. The nutrient agar medium contained 15 g of agar in addition to the above components.

For the isolation and/or purification of auxotrophs and transformants, Spizizen minimal medium,¹⁰⁾ supplemented with amino acids and/or bases, if necessary, was used.

For protoplast regeneration, HCP-1.5 medium containing 5 g of glucose, 5 g of casamino acids, 3.5 g of K_2HPO_4 , 1.5 g of KH_2PO_4 , 0.1 g of L-tryptophan, 15 g of polyvinylpyrrolidone (K90; mean molecular weight, 700,000; Nakarai Chemicals Ltd.), 1.9 g of $MgCl_2$ and 250 ml of 1.2 M sodium succinate (pH 7.3) per liter was used. HCP-1.5 agar medium comprised HCP-1.5 medium supplemented with 8 g of agar per liter. HCP-3 medium was the same as HCP-1.5 medium except that the polyvinylpyrrolidone was increased to 30 g per liter. Solutions of $MgCl_2$, sodium succinate and polyvinylpyrrolidone were autoclaved separately to prevent salt precipitation. Autoclaving a mixture of polyvinylpyrrolidone and agar prevented solidification of the medium.

Microorganisms. The strains used in this study are listed in Table I. Filamentous (*fil*) mutants were obtained from *Bacillus subtilis* AC327 as follows. 0.2 ml of a culture of *B. subtilis* AC327 grown in nutrient broth (5 ml) at 37°C for 14 hr was transferred to 20 ml of nutrient broth, followed by incubation at 37°C for 2 hr. The logarithmic-phase cells were collected by centrifugation and then suspended in 10 ml of 0.1 M Tris-maleate buffer (pH 6.0) containing 200 µg per ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in a 100-ml flask, followed by incubation at 37°C for 30 min. After centrifugation of the culture, the pellet was suspended in 1 ml of nutrient broth, and then a 0.1-ml portion of the suspension was transferred to 5 ml of nutrient broth. After incubation at 37°C for 14 hr, 0.2 ml of the culture was further transferred to 20 ml of nutrient broth to obtain a log-phase culture. After incubation at 37°C for 3 hr, 10 ml of the culture was centrifuged at $7,200 \times g$ for 10 min. Since we knew that the filamentous mutants, *lyt-1*, *lyt-2*, *rgn-1* and *rgn-2*, are apt to float up within a few minutes upon centrifugation under the above conditions, the centrifuge tube was slowly spun, and then the floating cells were pipetted out, diluted in nutrient broth and plated onto nutrient agar plates. The colonies that grew overnight at 37°C were examined for filamentation under a light microscope. To confirm filamentation, cells grown in a liquid culture were also examined.

Protoplast formation and transformation with gently lysed protoplasts (LP transformation). Protoplasts and competent cells were prepared as described by Akamatsu and Sekiguchi¹¹⁾ and Anagnostopoulos and Spizizen,¹²⁾ respectively. LP transformation was carried out as described previously.¹³⁾ 0.1 ml of a protoplast suspension was added to 1 ml of competent cells in a hypotonic buffer, followed by incubation at 37°C for 30 min. Then the cells were plated on minimal agar supplemented with appropriate nutrients. Each colony was further transferred to diagnostic agar plates and unselected marker(s) were determined.

Percent cotransformation was defined as (number of cotransformants per number of total transformants) $\times 100$.

Protoplast fusion and regeneration. The protoplast fusion method used was basically the same as that of Schaeffer *et al.*¹⁴⁾ Two protoplast suspensions, from different strains, were mixed and vortexed in SMM (0.5 M sucrose-0.02 M $MgCl_2$ -0.02 M maleate buffer, pH 6.5), followed by the addition of 40% polyethyleneglycol 4000 (average molecular weight, 3,000; Wako Pure Chem. Ind. Ltd.). After standing at 0°C for 1 min, the protoplast suspensions were diluted with HCP-1.5 medium.

The protoplast regeneration method was described previously.¹¹⁾ A 0.1-ml portion of the diluted protoplast suspension was placed on the surface of HCP-1.5 agar medium, followed by overlaying with 4 ml of the same medium containing agar. Colonies appearing on the regeneration agar medium were streaked on nutrient agar and then single colonies were examined for unselected markers.

Enzyme assays. The autolytic enzyme preparations and cell walls for the assays were prepared as described previously.⁶⁾ Log-phase cells were harvested and disrupted by ultrasonication. After centrifugation, the pellet was used as the cell wall fraction containing autolysins. The initial cell-wall concentration was always 0.2 at A_{540nm} and the percent decrease in A_{540nm} at 40°C in TK buffer (0.1 M Tris(hydroxymethyl)aminomethane, pH 8.0, containing 0.1 M KCl) was recorded.

Protease activity was determined as described previously.⁴⁾ One unit of protease activity was defined as the amount of the enzyme which solubilized 1 µg equivalent of tyrosine in 1 min.

Flagella staining, cell motility and morphology. The flagella of cells cultivated on nutrient agar at 37°C overnight were stained as described by Doetsch.¹⁵⁾ Cell motility was investigated both on nutrient agar containing 0.5% agar and under a phase-contrast microscope. Cell morphology was investigated at the logarithmic phase of growth in nutrient broth.

RESULTS AND DISCUSSION

Filamentous mutants (*e.g.*, *lyt* and *rgn*) tend to float up from pellets within a short time on high-speed centrifugation, and so we used this property for enrichment of filamentous mutants. Colonies grown on nutrient agar plates after plating of supernatants obtained on centrifugation were examined for filamentation. Candidates were transferred to nutrient broth and then incubated at 37°C until the growth reached the logarithmic phase. Filamentation of the cells was examined under a light microscope, and finally seven filamentous mutants (*fil-1* to *fil-7*) were isolated from different cultures.

Mapping of the fil-1 and fil-3 to fil-6 mutations through LP transformation

Autolysin-less mutants, *e.g.*, *lyt-1* and *lyt-2*, form filaments, and their genetic locus has been determined to be between *lys* and *aroD*.⁹⁾ These results prompted us to investigate the

linkage of *fil* mutations to the *aroD* locus. LP transformation of AC415 with AC551, AC553, AC554, AC555 and AC556 DNAs indicated that the *fil* mutations were linked to *aroD* at the frequency of 11.5% to 22.2% (Table II). As to congression, linkages of *aroD-leu-2* (map distance of 20°) and *aroD-lys* (16.5°) (from the linkage map in Fig. 1 of Piggot and Hoch, 1985)¹⁶⁾ were less than 2.8% and 5.6%, respectively. On the other hand, there was no significant linkage between *aroD* and *fil-2* or *fil-7* (data not shown).

Mapping of rgn mutations through protoplast fusion and LP transformation

Previous results indicated that *rgn* mutants also form filaments and contain a decreased amount of autolysin(s).⁶⁾ Since protoplast fusion is a convenient method for genetic mapping, protoplasts from a standard polyauxotrophic marker strain (AC343) were fused with ones from the *rgn-1* mutant (AC325). Ade⁺ fusants were selected on HCP-3 agar medium

TABLE II. LINKAGE RELATIONSHIPS OF *aroD* to *fil-1*, *fil-3*, *fil-4*, *fil-5* AND *fil-6* ON LP TRANSFORMATION

Donor ^a (genotype)	Recipient ^b (genotype)	Selection	Recombinant					Total No.
			Class					
			Lys ⁻ Leu ⁻ Fil ⁺	Lys ⁻ Leu ⁻ Fil ⁻	Lys ⁺ Leu ⁻ Fil ⁺	Lys ⁺ Leu ⁻ Fil ⁻	Lys ⁻ Leu ⁺ Fil ⁺	
AC551 (<i>purB his-1</i> <i>fil-1</i>)	AC415 (<i>lys aroD120</i> <i>asaA4 leu-2</i>)	Aro ⁺	72 ^c	12	1	2	1	88
AC553 (<i>purB his-1 fil-3</i>)	AC415	Aro ⁺	70	12	4	0	2	88
AC554 (<i>purB his-1 fil-4</i> <i>glu-1 com-1</i>)	AC415	Aro ⁺	52	14	2	2	2	72
AC555 (<i>purB his-1 fil-5</i>)	AC415	Aro ⁺	88	12	4	0	0	104
AC556 (<i>purB his-1 fil-6</i>)	AC415	Aro ⁺	66	12	2	0	0	80

^a The numbers of protoplasts used as donor DNAs from AC551, AC553, AC554, AC555 and AC556 were 2.0×10^4 , 8.3×10^3 , 6.2×10^3 , 1.4×10^4 and 7.3×10^3 , respectively.

^b The number of recipient cells was 7.4×10^7 .

^c The number of the class.

and the unselected markers were identified. The distribution of unselected marker classes is shown in Table III. The order of the markers

TABLE III. DISTRIBUTION OF UNSELECTED MARKER CLASSES AMONG ADE⁺ RECOMBINANTS OBTAINED ON PROTOPLAST FUSION BETWEEN *B. subtilis* AC343 (*strA met-1 glt-1 trpC2 leu-2 his-1 purA*) AND AC325 (*purB rgn-1*)^a

Number of colonies carrying indicated unselected markers						% Frequency of Rgn ⁻
Met	Glt	Trp	Leu	His	Number	
+	+	+	+	+	11	100 (11/11) ^b
-	+	+	+	+	48	100 (20/20)
-	-	+	+	+	10	100 (10/10)
-	-	-	+	+	5	60 (3/5)
-	-	-	-	+	8	0 (0/8)
-	-	-	-	-	8	0 (0/8)
-	-	-	+	-	1	
-	-	+	-	-	1	
-	+	-	-	+	2	
+	+	-	+	+	1	
-	+	+	-	+	1	
-	+	+	+	-	2	
-	+	-	+	-	1	
99						

^a The double, quadruple and sextuple cross-over classes comprise lines 1 to 6, 7 to 12, and 13, respectively.

^b In parentheses are the numbers of Rgn⁻ recombinants per number of recombinants examined in each class.

in the genetic map of *B. subtilis* was *strA-purB-met-1-glt-1-trpC2-leu-2-his-1-purA-strA* (*Mol. Gen. Genet.*, in press). The ratios of double, quadruple and sextuple cross-over recombinants to the total recombinants were 91%, 8% and 1%, respectively. Among the double cross-over recombinants, the cross-over between *trpC2* and *leu-2* led to 60% Rgn⁻. Cross-overs before the *trpC2-leu-2* region and after this region led to 100% and 0% Rgn⁻ phenotypes, respectively. These results clearly indicated that the *rgn-1* marker was located between *trpC2* and *leu-2*.

The *rgn-1* mutation was further mapped through LP transformation (Table IV). *rgn-1* linked to *dnaE* at a frequency of 29%, and *dnaE* to *aroD120* at frequencies of 36% to 39%. But *lys* did not link to *dnaE* or *aroD* on LP transformation. The three-factor cross in Table IV also indicates the order of *rgn-1-dnaE-aroD120*. Furthermore, the *rgn-2* mutation was found to be linked to the *aroD* locus (data not shown).

Since *lyt-1*, -2 and -152, *fil-1* and -3 to -6, *rgn-1* and -2 are located near *aroD*, whether or not these mutations are in the same locus was examined. LP transformation of AC708 (*lys rgn-1 dnaE20*) with AC620 (*rgn-2*), AC621 (*lyt-1*), AC622 (*lyt-2*), AC623 (*lyt-152*), AC601 (*fil-1*), AC603 (*fil-3*), AC604 (*fil-4*), AC605 (*fil-5*) and AC606 (*fil-6*) with *dnaE* selection

TABLE IV. LINKAGE RELATIONSHIPS OF *rgn-1* TO *aroD120* AND *dnaE* ON LP TRANSFORMATION

Donor ^a (genotype)	Recipient ^b (genotype)	Selection	Recombinant	
			Class	Number
168S (<i>strA trpC2</i>)	AC705 (<i>lys dnaE20 aroD120 asaA4</i>)	Ts ⁺ ^c	Aro ⁺ Lys ⁻	78
			Aro ⁻ Lys ⁻	122
		Aro ⁺	Ts ⁺ Lys ⁻	75
			Ts ⁻ Lys ⁻	125
AC703 (<i>lys rgn-1 leu-2</i>)	AC705	Ts ⁺	Aro ⁺ Rgn ⁻ Lys ⁻	15
			Aro ⁺ Rgn ⁺ Lys ⁻	23
			Aro ⁻ Rgn ⁻ Lys ⁻	16
			Aro ⁻ Rgn ⁺ Lys ⁻	52

^a The numbers of protoplasts of 168S and AC703 were 2.4×10^4 and 2.5×10^4 , respectively.

^b The number of recipient cells was 7.5×10^7 .

^c Temperature-independence, phenotype of *dnaE20*⁺.

yielded ratios of 0/96 (number of respectively).

Rgn⁺ transformants per number of total transformants), 0/103, 0/111, 0/1859, 2/1738, 1/2101, 0/78, 0/97 and 0/109, respectively. These results indicate that these mutations are in the same *flaD* locus (the *lyt-1* and -2 loci were recently designated as *flaD1* and *flaD2*,

Mapping of the *fil-2* and *fil-7* mutations through LP transformation

LP transformation of AC408 with the AC602 and AC607 protoplast lysates indicated a 25% linkage between *fil-2* and *pyrD*

TABLE V. LINKAGE RELATIONSHIPS OF *pyrD* TO *fil-2* AND *fil-7* ON LP TRANSFORMATION

Donor ^a (genotype)	Recipient ^b (genotype)	Selection	Recombinant	
			Class	Number
AC602 (<i>purB fil-2</i>)	AC408 (<i>pyrD his-1</i>)	Ura ⁺ ^c	Fil ⁻ His ⁻	23
			Fil ⁺ His ⁻	67
			Fil ⁻ His ⁺	0
			Fil ⁺ His ⁺	3
AC607 (<i>purB fil-7</i>)	AC408	Ura ⁺	Fil ⁻ His ⁻	15
			Fil ⁺ His ⁻	39
			Fil ⁻ His ⁺	0
			Fil ⁺ His ⁺	0

^a The numbers of protoplasts of AC602 and AC607 used as donor DNAs were 5.0×10^3 and 3.0×10^3 , respectively.

^b The number of recipient cells was 5.0×10^7 .

^c The numbers of Ura⁺ transformants were 513 and 287 on transformation of AC408 with the AC602 and AC607 lysates, respectively.

TABLE VI. COMPETENCY OF THE *rgn*, *lyt* AND *fil* STRAINS^a

Recipient ^b	Genotype	No. of transformants ^c	RTF ^d
AG5	<i>purB arg-15 rgn-1 leuB8</i>	1.7×10^4	4.9%
AH6	<i>purB6 arg-15 rgn-2 leuB8</i>	1.2×10^3	0.34%
AC621	<i>lys lyt-1 leu-2</i>	1.7×10^3	0.49%
AC622	<i>lys lyt-2 leu-2</i>	4.7×10^3	1.3%
AC623	<i>lys lyt-152 leu-2</i>	1.9×10^3	0.54%
AC601	<i>lys fil-1 leu-2</i>	9.2×10^3	2.6%
AC603	<i>lys fil-3 leu-2</i>	1.1×10^3	0.31%
AC604	<i>lys fil-4 leu-2 com-1</i>	<1	<0.0003%
AC790	<i>lys fil-4 leu-2</i>	2.3×10^2	0.07%
AC605	<i>lys fil-5 leu-2</i>	6.6×10^3	1.9%
AC606	<i>lys fil-6 leu-2</i>	4.5×10^3	1.3%
AC416	<i>trpC2 lys leu-2</i>	3.5×10^5	100%

^a LP transformation was carried out with *B. subtilis* 168S protoplast lysates. The number of protoplasts was 1.0×10^7 .

^b The same amounts (at $A_{660\text{nm}}$) of recipient cells were used for the transformation experiments, but the number of colony-forming units (CFU) was 1.0×10^7 except for the case of AC416 (1.0×10^8). Because the autolysin-deficient mutants are chain-forming (filamentous) mutants (average of 10 cell units per chain after incubation in the competent medium¹²). Since the transformation frequency of the "wild-type" strain with saturated DNA amounts was as low as 0.5%, it is considered that the numbers of transformants of the autolysin-deficient mutants were not affected by the chain formation.

^c The number of Leu⁺ transformants.

^d Relative transformation frequency.

and a 28% linkage between *fil-7* and *pyrD*, respectively (Table V). Congression estimated from the linkage between *pyrD* and *his-1* was less than 3%. Previous results indicated that linkage values of 32% and 16% on LP transformation correspond to ones of 76% and 55% on phage PBS1 transduction, respectively. Since two flagella-minus mutations (*flaA* and *flaB*) are located near *pyrD*, and the linkage values on PBS1 transduction were 69% and 46%, respectively,⁹ the *fil-2* and *fil-7* mutations may correspond to the *flaA* locus. Alternatively, the results may suggest a gene cluster of flagella mutations near the *pyrD* locus.

Properties of the *fil*, *rgn* and *lyt* mutations

It is known that some autolysin-minus mutants (i.e., *amyB* and *rgn*) lead to a decrease in

competency. Among the *fil* mutants, the *fil-2* and *fil-7* ones retained normal competency (data not shown). But the *fil-1* and *fil-3* to *-6* mutations in addition to the *lyt-1*, *lyt-2* and *lyt-152* ones led to a decrease in competency (Table VI). The ratio of the decrease ranged from 0.07% to 4.9%, except for in the case of the AC604 strain. Since the competency of AC604 was extremely low, it was considered that another mutation which affects competency had occurred during the NTG treatment.

When strain AC415 (*lys aroD120 asaA4 leu-2*) was transformed with DNA from AC604 (*lys fil-4 leu-2 com-1*) and Fil⁻ Aro⁺ Asa⁺ transformants were selected, all of the six transformants (e.g., AC790) examined showed similar competency levels to the other *flaD* mutants. Furthermore, when the recipient was changed to AC327 (*purB his-1*) and the same

FIG. 1. Phase Contrast Illuminated Photomicrography of the Parent, and *fil* and *rgn* Mutants after Flagella Staining.

Cells grown on the nutrient agar medium for 14 hr at 30°C were subjected to flagella staining as described under MATERIALS AND METHODS. Key: a, parent (AC327); b, *fil-1* (AC601); c, *fil-2* (AC609); d, *fil-3* (AC603); e, *fil-4* (AC604); f, *fil-5* (AC605); g, *fil-6* (AC606); h, *fil-7* (AC610); i, *rgn-1* (AG5). The bar represents 10 μ m.

transformation experiment was carried out, nine out of ten His⁺ Fil⁻ transformants retained competency at the *flaD* level, but one transformant exhibited extremely low competency (data not shown). These results suggest that both the *fil-4* mutation and a mu-

tation (designated as *com-1*) affecting competency are present in the AC604 strain, and the *fil-4* and *com-1* mutations are not closely linked. Genetic mapping and investigation of the properties of the *com-1* mutation are now in progress.

Flagella staining of the *fil* mutants in addition to the *rgn* one was carried out. Figure 1 shows that all of the *fil* and *rgn-1* mutants lacked flagella. The lack of motility of the mutant cells prepared from logarithmic-phase cultures supported the above result.

Fein reported that autolysin deficiency leads to a loss of flagella.¹⁷⁾ Thus, autolysin activities were measured for the *fil-2*, *fil-3*, *fil-6*, and *fil-7* mutants and a control strain, AC327. All of the *fil* mutants showed decreases in autolysin activity, ranging from 13% to 25% (Fig. 2).

Another interesting property among pleiotropic phenomena is extracellular enzyme productivity. When the α -amylase and protease amounts in *rgn* mutants were measured, the protease production by *rgn-1* and *rgn-2* mutants was found to be 2.0 and 3.0 times higher than that by YS11, respectively (Fig. 3). But α -amylase production by both mutants was not significantly different (unpublished results). Moreover, the time of initiation of protease production in the *rgn* mutants was two hours earlier than that in YS11.

A pleiotropic mutation, *sacQ*^h, which leads to higher production of extracellular enzymes and a decrease in competency did not cause a loss of flagella.¹⁸⁾ On the contrary, the hyperextracellular enzyme producing *sacU*^h (*amyB* and *pap*) mutation caused a decrease in competency and a lack of flagella.¹⁸⁾ Yang *et al.* reported that the *sacQ* gene product is a small 46-amino acid polypeptide and that the *sacQ*^h mutation leads to increased expression of this polypeptide.¹⁹⁾ Aubert *et al.* reported that the *sacU* gene product is a 46-kilodalton protein and that the *sacU*^h mutation leads to an increase in this protein in the membrane fraction.²⁰⁾ But Aymerich *et al.*²¹⁾ and Shimotsu and Henner²²⁾ reported that the *sacU*^h mutation enhances the transcription of *sacB* (the struc-

FIG. 2. Endogenous Autolytic Activities of the Parent and *fil* Mutants.

Symbols: ○, parent (AC327); □, *fil-3* (AC603); △, *fil-6* (AC606); ▽, *fil-2* (AC609); ■, *fil-7* (AC610).

FIG. 3. Time Courses of Cell Growth and Protease Production by the *rgn* Mutants.

Symbols: ○, parent (YS11); ●, *rgn-1* (AG-5); ▼, *rgn-2* (AH-6).

tural gene for an extracellular enzyme, levan-sucrase) and does not have an effect at secretion level. At present there is no explanation as to how the pleiotropic effect occurs.

On the basis of the results of this study, we could classify the autolysin- and/or flagella-deficient mutations into six groups as follows: (1) *lyt-1*, *lyt-2*, *lyt-152*, *fil-1*, *fil-3* to *fil-6*, *rgn-1* and *rgn-2* mutations in the *flaD* (219°) locus [phenotypes: flagella- and autolysin-deficient, competent-minus and filamentous]; (2) *lyt-15* in *flaA* (145°) [phenotypes: flagella- and autolysin-deficient, and rod-shaped]; (3) *fil-2* and *fil-7* linked to *pyrD* (135°) [phenotypes: flagella- and autolysin-deficient, normal competent and filamentous]; (4) *flaB* (149°) [phenotype: flagella-deficient; other properties are unknown]; (5) *flaC* (315°) [phenotype: flagella-deficient; other properties are unknown]; and (6) *lyt-151* linked to *hisA* (299°) [flagella- and autolysin-deficient, and filamentous].

The filamentous mutations will not only be useful for elucidation of the fundamental mechanism as to cell morphology and cell envelope architecture, but also for a better understanding of the pleiotropic effects caused by the *sacQ^h* and *sacU^h* mutations.

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