Molecular Cloning of a Gene Affecting the Autolysin Level and Flagellation in Bacillus subtilis

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(Received 22 September 1987; revised 25 January 1988)

A 2.8 kb PstI fragment of Bacillus subtilis 168W DNA has been cloned into Escherichia coli HB101 and B. subtilis AG5 using pAC3 as a shuttle plasmid. The new plasmid (pBRG1), of 10.2 kb, complemented flaD mutations which show reduced production of autolysin(s), filamentation and non-motility (deficiency of flagella). Deletion experiments showed that the suppressive gene is located between the HindIII and XbaI sites (1.0 kb apart) in pBRG1. The integration of a plasmid having chloramphenicol resistance closely linked to the flaD gene into the B. subtilis AC703 chromosome and its genetic analysis indicated that the cloned fragment contained the flaD gene itself. A high-copy-number plasmid carrying the cloned gene did not lead to an increase in autolysin production above the wild-type level, but it changed the colony morphology from smooth to rough. Among several autolysin-deficient mutations, lyt-151 was suppressed only by the high-copy-number plasmid carrying the cloned gene.

INTRODUCTION

The participation of bacterial autolysins in many important phenomena has been suggested by several authors (Tomasz, 1984; Pooley & Karamata, 1984a; Rogers et al., 1980). In Bacillus subtilis, autolysin(s) affect cell separation (Fein & Rogers, 1976), helical growth (Mendelson, 1982) and flagellation (motility) (Fein, 1979) in addition to cell lysis. Moreover, mutants which contain decreased amounts of autolysin(s) sometimes exhibit reduced competence for transformation (Ayusawa et al., 1975; Akamatsu & Sekiguchi, 1987c) and more frequent regeneration of protoplasts (Akamatsu & Sekiguchi, 1983b).

B. subtilis produces two autolysins, N-acetylmuramyl-l-alanine amidase (amidase) and endo-β-N-acetylglucosaminidase (glucosaminidase), the former being a major autolysin. Recently, a bacterial amidase gene was cloned from Streptococcus pneumoniae (Garcia et al., 1985) and sequenced (Garcia et al., 1986), and a lyt-4 mutant was determined to be deficient in amidase (Garcia et al., 1985). In the case of Bacillus, it has not been established whether the autolysin-deficient mutants reported up to now are affected in the structural gene or regulatory gene of the corresponding amidase or glucosaminidase. The autolysin-deficient phenotypes determined by some of the mutations previously described were obviously caused indirectly, e.g. teichoic acid mutation (Forsberg et al., 1973) and sacU mutation (Ayusawa et al., 1975).

Results obtained by Pooley & Karamata (1984b) and by us (Akamatsu & Sekiguchi, 1987a, c) indicate the existence of the following groups of autolysin-deficient mutations: (1) lyt-1, lyt-2, lyt-152, rgn-1, rgn-2, fil-1 and fil-3 to fil-6, which are located in flaD and cause both filamentation and deficiency of flagella; (2) the lyt-I5 (new designation flaA15) mutation, which is linked to pyrD and causes deficiency of flagella but does not affect cell size; (3) fil-2 and fil-7, which are

Abbreviation: LP transformation, lysed-protoplast transformation.

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also linked to pyrD and cause both filamentation and deficiency of flagella; and (4) the ltr-151 mutation, which is linked to hisA and causes both filamentation and deficiency of flagella.

In this report, we describe the cloning of a B. subtilis DNA fragment which suppresses flaD mutations in B. subtilis, the characterization of the cloned fragment, and the properties of transformants harbouring a high copy number of the plasmid carrying the cloned gene.

METHODS

Bacterial strains and plasmids. These are listed in Table 1. pJKK310 was a shuttle plasmid (10·5 kb), between E. coli and B. subtilis, constructed by ligation of EcoRI-cleaved pBR325 and pUB110; it led to the expression of KmR ApR TcR in E. coli and KmR in B. subtilis. pAC3 was a deletion plasmid (7·4 kb) derived from pJKK310; it led to the expression of KmR TcR in both E. coli and B. subtilis. The 3·1 kb deletion, between the tet gene and the kan gene, caused readthrough by RNA polymerase from the kan promoter, leading to the expression of TcR. The construction of the other plasmids is described in the text and Fig. 4.

Media. For bacterial growth, nutrient broth [10 g beef extract (Difco), 10 g Bacto-peptone (Difco) and 2 g NaCl per litre (pH 7·0)] was used. For isolation of transformants, Spizizen minimal medium (Spizizen, 1958) was used as a basal medium. For preparation of competent broth of E. coli, L broth [10 g tryptone (Difco), 5 g yeast extract (Difco), 1 g glucose and 5 g NaCl per litre (pH 7·2)] was used. For regeneration of B. subtilis protoplasts, HCP-1·5 medium (Akamatsu & Sekiguchi, 1984) [5 g glucose, 5 g Casamino acids, 3·5 g K2HPO4, 1·5 g KH2PO4, 0·1 g L-tryptophan, 15 g polyvinylpyrrolidone, 1·5 g MgCl2, and 250 ml 1·2 M-sodium succinate per litre (pH 7·3)] was used. HCP-1·5 agar medium was HCP-1·5 medium supplemented with 8 g agar 1−1. HCP-3 medium was the same as HCP-1·5 medium except that the amount of polyvinylpyrrolidone was increased to 30 g 1−1. Solutions of MgCl2, sodium succinate and polyvinylpyrrolidone were autoclaved separately to prevent salt precipitation. Autoclaving of a mixture of polyvinylpyrrolidone and agar prevented solidification of the medium.

Preparation of B. subtilis protoplasts. Protoplasts were prepared as described previously (Akamatsu & Sekiguchi, 1983a, 1984) by incubation in SMM (0·5 M-sucrose/0·02 M-MgCl2/0·02 M-maleate buffer, pH 6·5) containing 250 µg lysozyme (Sigma) ml−1 at 42 °C for 45 min.

Transformation. Transformation of E. coli was done as described by Mandel & Higa (1970). Conventional transformation of B. subtilis was done according to the procedure of Anagnostopoulos & Spizizen (1961); protoplast transformation was done as described previously (Akamatsu & Sekiguchi, 1984).

Lysed-protoplast (LP) transformation of B. subtilis. This was done as described previously (Akamatsu & Sekiguchi, 1987b). A 0·1 ml volume of the protoplast suspension was added to 1 ml of a hypotonic competent cell suspension, followed by incubation at 37 °C for 30 min. Then the cells were plated out on minimal agar medium supplemented with appropriate nutrients. Percentage cotransformation was defined as (number of cotransformants per number of total transformants) × 100.

Preparation of plasmids. Large-scale preparation of plasmids from E. coli and small-scale preparation from E. coli or B. subtilis were done according to the procedures of Guerry et al. (1973) and Birnboim & Doly (1979), respectively.

Gel electrophoresis. Plasmid DNA was analysed on vertical or submerged 1% (w/v) agarose (type LO-3; Takara Shuzo Co.) gels, as described by Sharp et al. (1973). Low-molecular-mass DNA fragments were separated on 8% (w/v) acrylamide gels, and purification of DNA with low-melting-point agarose (Type ER; Takara Shuzo Co.) was done as described by Maniatis et al. (1982).

DNA manipulations. The probe was labelled with a nick-translation kit containing [α-32P]dCTP (Amersham). DNA fragments which had been separated by electrophoresis on 1% agarose gel were transferred to a Biodyne transfer membrane (Pall) by the method of Southern (1975). Hybridization was done in 3 × SSC (1 × SSC is 0·15 M-NaCl/0·015 M-trisodium citrate, pH 7·0) at 65 °C with a final wash at the same temperature in 3 × SSC. Autoradiography was done using Fuji RX X-ray film with Fuji intensifying screens at −80 °C. The standard recombination technique described by Maniatis et al. (1982) was used.

Gene library of B. subtilis chromosomal DNA in E. coli. B. subtilis 168W chromosomal DNA (10 µg), isolated as described by Saito & Miura (1963) and digested with PstI, was mixed with PstI-digested pAC3 DNA (2·5 µg) and then ligated with T4 DNA ligase at 18 °C for 16 h. The ligated DNA solution was added to competent cells of E. coli HB101, and then KmR ApR transformants were selected on L agar plates containing kanamycin and/or ampicillin, which yielded a gene library of 2700 colonies. The library was divided into 50 fractions and preserved in 50% (v/v) glycerol at −80 °C. Hybrid plasmid DNAs were prepared from cultures inoculated with the fractions.

Assay of autolysin(s). The autolytic enzyme preparation, and cell walls for the assays, were prepared as described previously (Akamatsu & Sekiguchi, 1983b).

Staining of flagella, and observation of cell motility and morphology. The flagella of cells cultivated on nutrient agar at 37 °C overnight were stained as described by Doetsch (1981). Cell motility was investigated both on nutrient agar containing 0·5% agar and under a phase-contrast microscope. Cell morphology was observed during the
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Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Derivation, source or reference</th>
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</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>proA2 leuB6 thi-1 lacY1 hsdR hsdM recA13 supE44 rpsL20</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td>HB101</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>trpC2</td>
<td>This laboratory</td>
</tr>
<tr>
<td>168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168S</td>
<td>trpC2 strA smo-1*</td>
<td>Akamatsu &amp; Sekiguchi (1983a)</td>
</tr>
<tr>
<td>168W</td>
<td>strA smo-1</td>
<td>Akamatsu &amp; Sekiguchi (1987a)</td>
</tr>
<tr>
<td>YS11</td>
<td>purB6 arg-15 leuB8</td>
<td>Akamatsu &amp; Sekiguchi (1987c)</td>
</tr>
<tr>
<td>196</td>
<td>trpC2 amyB</td>
<td>Sekiguchi et al. (1975)</td>
</tr>
<tr>
<td>AG5</td>
<td>purB6 arg-15 rgn-1 leuB8</td>
<td>Akamatsu &amp; Sekiguchi (1983b)</td>
</tr>
<tr>
<td>AC310</td>
<td>purB tet-1 smo-1</td>
<td>Transformation of AC327 with 196 DNA</td>
</tr>
<tr>
<td>AC327</td>
<td>purB his-1 smo-1</td>
<td>Akamatsu &amp; Sekiguchi (1987a)</td>
</tr>
<tr>
<td>AC330</td>
<td>his-1 amyB smo-1</td>
<td>Transformation of AC327 with 196 DNA</td>
</tr>
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<td>AC335A</td>
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<td>Akamatsu &amp; Sekiguchi (1987b)</td>
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<td>AC552</td>
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</tr>
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<td>AC628</td>
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<td>Transformation of AC310 with AC552 DNA</td>
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<td>lys dnaE20 aroD120 asaA4 smo-1</td>
<td>Akamatsu &amp; Sekiguchi (1987c)</td>
</tr>
<tr>
<td>AC708</td>
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<td>Transformation of AC703 with pBRG10 DNA</td>
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<td>AC780A</td>
<td>lys rgn-1 cat leu-2 smo-1</td>
<td>Akamatsu &amp; Sekiguchi (1987c)</td>
</tr>
<tr>
<td>AC780B</td>
<td>lys rgn-1 cat leu-2 smo-1</td>
<td>Transformation of AC703 with pBRG9 DNA</td>
</tr>
<tr>
<td>AC801</td>
<td>purB</td>
<td>Transformation of AC327 with 168 DNA</td>
</tr>
</tbody>
</table>

| Plasmids | | |
| pUC12 | Ap<sup>R</sup> Lac<sup>+</sup> | Messing (1983) |
| pUC18 | Ap<sup>R</sup> Lac<sup>+</sup> | Yanisch-Perron et al. (1985) |
| pUC19 | Ap<sup>R</sup> Lac<sup>+</sup> | Kreft et al. (1983) |
| pJKK310 | Ap<sup>R</sup> Te<sup>R</sup> Km<sup>R</sup>; replicates in *E. coli* and *B. subtilis* | |
| pC194 | Cm<sup>R</sup>; replicates in *B. subtilis* (and *E. coli*) | Ehrlich (1978) |
| pHW9 | Cm<sup>R</sup> Ap<sup>R</sup>; replicates in *E. coli* | Horinouchi & Weisblum (1982) |

* Smooth colony. Recently we observed a difference in colony type between 168 and 168S. Strain 168S and isogenic strains derived from it showed smooth colonies. The smo<sup>-1</sup> gene was linked to his<sup>A</sup> on LP transformation (data not shown).

Exponential phase of growth in nutrient broth. If necessary, kanamycin was added to a final concentration of 5 μg ml<sup>-1</sup>.

Enzymes. All restriction endonucleases, except for *PstI* (Takara Shuzo Co.), were purchased from Nippon Gene Co. T4 DNA ligase and RNAase A were purchased from Takara Shuzo Co. and Sigma, respectively.

**RESULTS**

Cloning of a gene which suppresses the Fla<sup>-</sup> phenotype

Protoplasts from the motility-minus pleiotropic mutant *B. subtilis* AG5 were transformed with DNA from the gene library, and colonies that appeared on HCP-1-5 agar plates containing...
kanamycin were collected and spotted onto soft agar plates for the detection of motility. Among 2700 transformant clones, one was motile with a typical rod morphology (Fig. 1) and harboured a 10-2 kb plasmid (pBRG1) that expressed KmR and TcR in both E. coli and B. subtilis. Plasmid pBRG1 carried a 2-8 kb inserted fragment with several restriction sites (Fig. 2). To confirm that the inserted DNA was a fragment of the B. subtilis chromosomal DNA, it was subjected to Southern blot analysis. Chromosomal DNA was digested with PstI, EcoRI and HindIII and the blots were probed with 32P-labelled pBRG1 (Fig. 3). Hybridization was observed with a fragment of each of the endonuclease digests (PstI, EcoRI and HindIII), and the fragment sizes of 2-8, 2-6 and 1-85 kb were similar to those obtained by digestion of pBRG1 with the same restriction enzymes. The weak hybridization evident with a faster-migrating fragment in lane 6 of Fig. 3 suggests the presence of a HindIII site at a distance of 1-6 kb from one of the HindIII sites in Fig. 2. These data suggest that no rearrangement of the cloned chromosomal DNA fragment occurred.

To confirm that pBRG1 possessed the complete DNA fragment needed to suppress the flaD mutation, a Fla+ transformant of B. subtilis AG5, with pBRG1 DNA, was inoculated into nutrient broth and then incubated at 37 °C for 15 generations. The culture was plated on nutrient agar plates, and growing colonies were replica-plated onto agar media with or without kanamycin. Eighty-four percent of the colonies were both KmS and had lost their motility. Plasmid analysis of six randomly chosen KmS colonies showed that they did not harbour any plasmids and confirmed the loss of the pBRG1-determined phenotype and reversion to the phenotype determined by the flaD mutation. The inserted fragment was reduced by deletion and subcloning to the minimum size required for complementation of the mutant phenotype (Fig. 2). Deletion of a 1-85 kb HindIII fragment from pBRG1 led to the formation of pBRG4. pBRG6 was also derived from pBRG1 by insertion of the 1-0 kb XbaI fragment of B. subtilis chromosomal DNA at a XbaI site. pBRG7 was derived from pJKK310 by replacing its 5-1 kb PstI–XbaI fragment by the 1-6 kb PstI–XbaI fragment of pBRG1 (Fig. 4). pBRG8 was a pAC3 derivative having the 1-55 kb EcoRI–XbaI fragment of pBRG1 (Fig. 4). pBRG13 was a pAC3 derivative having the 1-0 kb HindIII–XbaI fragment of pBRG1 (Fig. 4). Transformation of B. subtilis AG5 with these plasmids showed that all of them suppressed the Fla- phenotype except for pBRG4. Moreover, pBRG5, whose 1-85 kb HindIII fragment was in the reverse orientation compared to pBRG1, also suppressed the Fla- phenotype. These results suggested that the gene which suppressed the flaD mutation was located in the 1-0 kb HindIII–XbaI fragment (Fig. 2).

Identification of the cloned DNA in the B. subtilis chromosome

Previous work indicated that rgn mutations (rgn-1 and rgn-2), some autolysin-minus mutations (lyt-1, lyt-2 and lyt-152) and some filamentous mutations (fil-1, fil-3, fil-4, fil-5 and fil-6) are all located in the flaD locus (Akamatsu & Sekiguchi, 1987a). When strains, each having one of the mutations, were transformed with pBRG8 (KmR TcR in B. subtilis), all of the KmR
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**Fig. 2.** Physical map of pBRGl (a) and localization of the suppressive function (b). (a) Hatched and open blocks represent fragments of pUB110 DNA and B. subtilis chromosomal DNA, respectively. Arches represent DNA fragments of pBR325. (b) The cloned B. subtilis chromosomal DNA fragments only are presented. pBRG6 is a plasmid in which a 1 kb fragment of B. subtilis chromosomal DNA was inserted at a XbaI site. The construction of the other plasmids is described in the text and Fig. 4. These plasmids were introduced into B. subtilis AG5 and the suppressive function was scored as recovery of motility and loss of filamentation: +, suppression (cells motile and rod-shaped); –, non-suppression (cells non-motile and filamented). The numbers represent the distances (kb) between adjacent restriction sites.

Transformants were motile and rod-shaped. This supported the above finding that pBRGl possessed an entire DNA fragment which suppressed flaD mutations.

To determine the relationship of this fragment with flaD, an integrative plasmid (lacking ori for B. subtilis), pBRG10, which consists of the cat gene originally derived from pC194, the 1-0 kb HindIII–XbaI fragment of pBRGl and a part of pUC12 DNA (Fig. 4), was used as a donor DNA for protoplast transformation. CmR transformants of B. subtilis AC703 were isolated, and their motility and cell morphology were examined. Out of 29 CmR transformants, 28 were motile and rod-shaped, and did not harbour plasmids. These results indicate that the cat gene was integrated into the B. subtilis chromosome. Genetic mapping by LP transformation of AC703 with protoplasts from one of the transformants (AC703A) indicated that CmR was linked to dnaE20 with 26% cotransformation, while cotransformation in the case of CmR to flaD was 100% (Table 2). Since the linkage between dnaE20 and rgn-l (a mutation in the flaD locus) was previously reported to be 29% (Akamatsu & Sekiguchi, 1987c), the integrative site of the cat gene was considered likely to be at the flaD locus. These data suggest that the cloned fragment contained the flaD gene, which had been recombined in the homologous flaD locus.

To confirm the above conclusion, another integrative plasmid, pBRG9 (Fig. 4), which contains the cat gene from pC194 and a 0-6 kb Psrl–HindIII fragment closely linked to the
2.32
2.02
0.56

Fig. 3. Southern transfer of restriction-enzyme-digested DNAs from B. subtilis 168W and hybridization with pBRG1 as a probe. Lane 1, HindIII-digested λ DNA; lanes 2 and 5, EcoRI-digested 168W chromosomal DNA; lanes 3 and 6, HindIII-digested 168W chromosomal DNA; lanes 4 and 7, PstI-digested 168W chromosomal DNA. Lanes 1 to 4, agarose gel electrophoresis of the DNAs; lanes 5 to 7, blots with 32P-labelled pBRG1. The hybridization conditions are given in Methods. The sizes (kb) of the λ HindIII fragments are shown on the left.

'suppressive gene' in pBRG1, was used as a donor DNA for protoplast transformation of B. subtilis AC703. Among 25 CmR transformants, two (AC780A and AC780B) were randomly picked out. Both transformants did not harbour plasmids, and they were both non-motile and filamentous. Transformation of B. subtilis AC705 with protoplast lysates from AC780A and AC780B indicated that CmR was completely linked to rgn-1, the linkage of CmR to dnaE being 38% or 46%, and the gene order being cat–dnaE–aroD120 (Table 2). Thus the cloned fragment is considered to contain the flaD gene.

Properties of B. subtilis strains harbouring flaD plasmids

Since flaD is a pleiotropic mutation, one of its other effects, that on autolysin production, was measured in B. subtilis AG5(pBRG1) and B. subtilis YS11(pBRG1), and in control strains, AG5(pAC3) and YS11(pAC3) (Fig. 5). While the autolytic activity of AG5(pAC3) was about 30% of that of YS11(pAC3), the activity of AG5(pBRG1) was restored to the wild-type [YS11(pAC3)] level. The activity of YS11(pBRG1) was also similar to the wild-type level.

Differences in motility and colony morphology depending on the copy number of the flaD gene

When autolysin-deficient mutants, B. subtilis AC335A (lyt-151), AC330 (amyB) (Sekiguchi et al., 1975; Steinmetz et al., 1976), AC602 (fil-2) and AC607 (fil-7), were transformed with an integrative plasmid, pBRG10, CmR transformants did not show changes in their properties (e.g. motility and cell shape). However, when a high-copy-number plasmid, pBRG8 (50 copies of which are assumed to be present in B. subtilis, because it contains a PUB110 replicon: Gryczan et al., 1978), was introduced into the mutants, only lyt-151 was suppressed, and the resultant
Fig. 4. Genealogy and construction of plasmids. The V-hatched sequences are those from the B. subtilis chromosomal DNA which was originally cloned in B. subtilis AG5. Open blocks in pUD1, pBRG9 and pBRG10 indicate the 0.9 kb fragment of pHW9 which contains the chloramphenicol acetyltransferase (cat) gene and which was originally derived from a fragment of pC194. Relevant restriction endonuclease sites only are indicated. The numbers around the plasmid circles indicate the distance in kb from the basal point. The arrows indicate the direction of transcription and appropriate boundaries of the structural genes. Asterisks indicate integrative plasmids for B. subtilis. Multiple cloning site regions are expanded and drawn outside the circles. Ap, ampicillin resistance; Tc, tetracycline resistance; Km, kanamycin resistance; Cm, chloramphenicol resistance; lac, β-galactosidase structural gene; B, BamHI; C, ClaI; E, EcoRI; H, HindIII; Ha, HaeIII; Mb, MboI; Ms, MspI; P, PstI; S, SmaI; T, TaqI; X, XbaI.
Fig. 5. Autolytic activities of *B. subtilis* AGS(pBRG1) (○), AGS(pAC3) (●), YS11(pBRG1) (△) and YS11(pAC3) (▲).

Table 2. *Linkage relationships of integrated antibiotic markers to standard markers, determined by LP transformation*

<table>
<thead>
<tr>
<th>Donor (genotype)</th>
<th>Recipient (genotype)</th>
<th>Selected marker phenotype</th>
<th>Transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC703A (lys leu-2 rgn-1 : cat smo-1)</td>
<td>AC708 (lys rgn-1 dnaE20 smo-1)</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt; Leu Ts&lt;sup&gt;−&lt;/sup&gt; Rgn&lt;sup&gt;−&lt;/sup&gt;</td>
<td>32</td>
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<td>AC703A (lys leu-2 rgn-1 : cat smo-1)</td>
<td>AC708 (lys rgn-1 dnaE20 smo-1)</td>
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<td>AC780B (lys leu-2 rgn-1 cat smo-1)</td>
<td>AC705 (lys dnaE20 aroD120 asaA44 smo-1)</td>
<td>Cm&lt;sup&gt;−&lt;/sup&gt; Aro Ts&lt;sup&gt;−&lt;/sup&gt; Rgn&lt;sup&gt;−&lt;/sup&gt;</td>
<td>26</td>
</tr>
</tbody>
</table>

*The dnaE mutant is temperature-sensitive. Temperature sensitivity was examined at 48 °C (non-permissive) and 30 °C (permissive): +, wild-type; −, temperature-sensitive.*
AC335A(pBRG8) was a motile rod. The colony morphology of AG5 and AC801 was also changed from rough to smooth on the introduction of multiple flaD genes, but the smooth phenotype of AC335A, AC330, AC602 and AC607 was not changed. The map positions of lyt-151 and smo-1 were different from that of flaD. Moreover, the introduction of pBRG8 into AC330, AC602 and AC607 did not significantly increase the autolysin amounts, but its introduction into AC335A restored autolysin(s) to the wild-type level (data not shown). Therefore it would be interesting to determine how the high copy number of the flaD gene affects lyt-151 mutation and colony morphology.

**DISCUSSION**

The inactive form (E-form) of pneumococcal amidase is a monomer of \( M, \) 35000 as determined by SDS-polyacrylamide gel electrophoresis (Holtje & Tomasz, 1976); cloning and sequencing of its gene support the above result (calculated \( M, \) 36532: García et al., 1985, 1986). The *B. subtilis* amidase and glucosaminidase were shown to be a monomer and a dimer, respectively, in LiCl buffer (Rogers et al., 1984). The \( M, \) of the amidase is 30000–40000 (Rogers et al., 1984) or 50000 (Herbold & Glaser, 1975), and the subunit \( M, \) of the glucosaminidase is 90000 (Rogers et al., 1984). Our experiments indicate that a 1-0 kb fragment contained the flaD gene (Fig. 2). If this fragment coded for a protein, glucosaminidase would be eliminated as a candidate for the flaD gene product. Moreover, the introduction of a high copy number of the plasmid containing flaD to a flaD+ strain (YS11) did not lead to increases in autolysin(s) (Fig. 5). In contrast, flaD1 and flaD2 mutations led to decreases in both amidase and glucosaminidase, only 5% of the wild-type levels being retained (Fein, 1979; Fein & Rogers, 1976). Thus the flaD mutation probably does not involve the structural gene for amidase.

Autolysin-deficient mutants of Gram-positive bacteria (i.e. *Streptococcus faecalis*, *Staphylococcus aureus* and *B. subtilis*) often form cell chains or clumps (Shungu et al., 1979; Chatterjee et al., 1976; Fein & Rogers, 1976), but those of Gram-negative bacteria (i.e. *E. coli*) show no morphological differences (Tomioka et al., 1983). However, we could not strictly compare them, because it is not known whether they are the same type of mutation. In *B. subtilis*, the flaD mutation led to a loss of flagella (Fein, 1979) and a decrease in competence (Pooley & Karamata, 1984a; Akamatsu & Sekiguchi, 1987c). To classify the autolysin-deficient mutations and to investigate the properties and regulation of autolysin(s), the cloned fragment in pBRG1 will be very important. This fragment is currently being used to determine the nucleotide sequence of the flaD gene.

Our stock strain, *B. subtilis* 168S (trpC2 strA smo-1), and its derivatives formed smooth colonies. Since the 168 strain was rough, the morphological change was examined. The smooth mutation (smo) in *B. subtilis* has already been reported by Grant & Simon (1969) and Karamata et al. (1972), who indicated the linkage of smo to hisA. Our LP-transformation experiments indicated the linkage of the smo-1 marker to his-1 (his-A) (data not shown). These data suggest that they are the same type of mutation. Since only a high copy number of the flaD gene in *B. subtilis* changed the phenotype from smooth to rough, it will be interesting to elucidate the mechanisms of the flaD effect.

We wish to thank Dr H. Moriyama for the technical assistance in the Southern hybridization. This work was supported in part by a Grant-in-Aid for Scientific Research from the Mishima Kaiun Memorial Foundation.

**REFERENCES**


Cloning of a gene affecting autolysin


