Incorporation of the Whole Chromosomal DNA in Protoplast Lysates into Competent Cells of Bacillus subtilis

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Competent cells of Bacillus subtilis AC870 (purB, leuB, trpC, ald-I) were transformed to Ade', Trp', or Ade' Trp' with DNA in protoplast lysates of B. subtilis AC819 (hisH, tet-1, rpsL, smo-I). The cotransfer ratio of purB to trpC was constant at 7-9% (Ade' Trp' /Trp') or 3% (Ade' Trp' /Ade') at protoplast concentrations of 2.7×10^7 to 2.7×10^9 per ml. The whole chromosomal DNA must be certainly incorporated into competent cells from the following reasons; (1) purB is opposite to trpC on the chromosome, (2) 2.7×10^7 protoplasts per ml is about 100 times lower than 3.2×10^9 competent cells per ml, and (3) the cotransfer ratio is constant at all the concentrations. Similar results were observed with the cotransfer ratio of purA to trpC. The transformation requires several Com proteins including ComK.

Key words: Bacillus subtilis; transformation; competence; DNA; protoplast

Natural transformation in bacteria has been thoroughly studied using both purified DNA and the recipient cells in a physiological state known as competence. In Bacillus subtilis, double-stranded donor DNA is bound to the competent recipient cell surface. Limited cleavage of the DNA by nuclease occurs, yielding fragments of 10×10^6 to 20×10^6 daltons (15 kb - 30 kb). About half of the DNA is degraded by a competence-specific nuclease and released into the medium as acid-soluble degradation products. The remaining single-stranded DNA is then transported into the cytosol and interacts with recipient DNA to form joint molecules which are joined by noncovalent bonds. In the joint molecule, the donor moiety has replaced an equivalent segment of recipient DNA. The joint molecule is then covalently sealed, yielding a heteroduplex recombinant molecule. Finally, expression of donor genetic information, segregation of the heteroduplex, and replication occur, resulting in the formation of a transformant clone.

A large number of proteins required for transformation have been identified in Bacillus subtilis. All are regulated at the transcriptional level and depend for their synthesis on the comK transcriptional factor. ComG and ComC proteins are the DNA-binding apparatus. ComE and ComF proteins are the DNA transport machinery. An attractive working hypothesis has been proposed for the transformation process and for other Com proteins of B. subtilis.

The direct use of DNA in L-form lysates or protoplast lysates leads to high transformation efficiency. Transformants are obtained at a frequency of one transformant per ten genomes, 200-1,000 times higher than the conventional method using purified DNA. Moreover, significantly high cotransformation linkages have also been observed between two markers separated by a distance corresponding to 2.3% (about 100 kb) of the total B. subtilis chromosome, using DNA in protoplast lysates. The high linkage relationship cannot be observed with purified and fragmented DNA. Chromosomal DNA in protoplasts is intact since the B. subtilis protoplasts prepared by lysozyme treatment are regenerated at the frequency of 92 - 100%. In spite of these advantages, little is known about the transformation using DNA in protoplast lysates.

In this study we describe the incorporation of the whole chromosomal DNA into a competent cell of B. subtilis and involvement of Com proteins in the transformation with DNA in protoplast lysates of B. subtilis.

Materials and Methods

Bacterial strains and plasmid. The bacterial strains and plasmid used in this study are listed in Table 1. Plasmid pCB1 was constructed by blunt-ending the HindIII site of pC194 with the Klenow fragment and cloning the resulting DNA into the Nael site of pBlueScript II sk (+). The plasmid is a shuttle one (5,875 bases) between Escherichia coli and B. subtilis and leads to the expression of Ap-r Cm-r in E. coli and Cm-r in B. subtilis.

Media. Luria-Bertani medium (LB medium) was

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used for bacterial growth.\textsuperscript{14} The LB agar medium contained 15 g of agar in 1 liter of LB medium. Spizizen minimal medium (SM medium)\textsuperscript{15} was used as a basal medium for preparation of competent cells. For isolation of auxotrophs or transformants, low Spizizen minimal medium (LSM medium) if necessary, supplemented with amino acids, was used.\textsuperscript{14}

Preparation of \textit{B. subtilis} protoplasts. Protoplasts were prepared basically as described by Akamatsu and Sekiguchi\textsuperscript{12,13} by incubation in SMM (0.5 M sucrose-0.02 M maleate buffer, pH 6.5-0.02 M MgCl\textsubscript{2}) containing 250 μg lysozyme (Sigma) per ml at 40.5°C for ~45 min. After incubation, 3 ml of the suspension was transferred to a centrifuge tube containing 10 ml of SMM and the tube was centrifuged (4,000 × g for 5 min). The pellet was suspended in SMM buffer. Protoplast density was measured by absorbance at 660 nm (A\textsubscript{660}). The 2.88 of A\textsubscript{660} corresponds to 2.74 × 10\textsuperscript{4} protoplasts per ml. After dilution with SMM buffer, a portion (0.1 ml) of protoplast suspension was used as the source of donor DNA.

\textbf{Transformation with DNA obtained by gentle lysis of protoplasts (LP transformation).} Competent cells were prepared basically as described by Anagnostopoulos and Spizizen.\textsuperscript{21} LP transformation was done as described previously.\textsuperscript{12} A portion (0.1 ml) of a protoplast suspension was added to 1 ml of competent cell culture and incubated at 37°C for 30 min. Then the cells were plated out onto minimal agar with appropriate nutrients. Each colony, if necessary, was further transferred to diagnostic agar plates and unselected marker(s) were identified. In the case of plasmid transformation, 1 ml of LB medium was added to each tube with the 1.1 ml of transformation mixture, and the tubes were moreover incubated at

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Bacterial strain and plasmid} & \textbf{Genotype or phenotype} & \textbf{Reference, source or derivation} \\
\hline
\textit{B. subtilis} & trpC2 rpsL smo-1 & Akamatsu and Sekiguchi\textsuperscript{16} \\
168S & trpC2 aroG932 leuB8 ald-1 & Akamatsu and Taguchi\textsuperscript{14} \\
QB936 & tet-1 rpsL hisH smo-1 & Akamatsu et al\textsuperscript{15} \\
AC819 & purB hisH smo-1 & Akamatsu and Sekiguchi\textsuperscript{16} \\
AC327 & purB smo-1 & Tfm (168S: AC327 DNA, Trp\textsuperscript{+} Ade\textsuperscript{-} Str-s)\textsuperscript{b} \\
AC710 & rpsL smo-1 purA & Akamatsu et al\textsuperscript{15} \\
AC843 & purB trpC2 leuB8 ald-1 & Tfm (QB936: AC710 DNA, Ara\textsuperscript{+} Ade\textsuperscript{-}) \\
AC870 & purA trpC2 leuB8 ald-1 & Tfm (QB936: AC843 DNA, Ara\textsuperscript{+} Ade\textsuperscript{-}) \\
AC871 & trpC2 leuB8 ald-1 & Tfm (QB936: 168S DNA, Ara\textsuperscript{+}) \\
1A706 & com-9 hisA1 leu-8 lys-21 metB5 thr-5 trpC2 & \textsuperscript{a} \\
1A708 & comK hisA1 leu-8 lys-21 metB5 thr-5 trpC2 & b \\
1A709 & comM hisA1 leu-8 lys-21 metB5 thr-5 trpC2 & b \\
1A710 & com31 hisA1 leu-8 lys-21 metB5 thr-5 trpC2 & b \\
1A711 & com14::Tn917 hisA1 leu-8 lys-21 metB5 purB6 thr-5 trpC2 & b \\
1A713 & comR::Tn917 hisA1 leu-8 lys-21 metB5 purB6 thr-5 trpC2 & Tfm (QB936: 1A711DNA, Em-r) \\
ST1 & com14::Tn917 aroG932 leuB8 trpC2 ald-1 & Tfm (QB936: 1A713DNA, Em-r) \\
ST3 & comR::Tn917 aroG932 leuB8 trpC2 ald-1 & Tfm (QB936: ST1DNA, Em-r) \\
KUS10 & com14::Tn917 aroG932 leuB8 trpC2 ald-1 & Tfm (QB936: ST3DNA, Em-r) \\
KUS11 & com9 leuB trpC2 ald-1 & Tfm (QB936: 1A706DNA, Ara\textsuperscript{+} Com\textsuperscript{+}) \\
KUS12 & comK leuB trpC2 ald-1 & Tfm (QB936: 1A709DNA, Ara\textsuperscript{+} Com\textsuperscript{+}) \\
KUS13 & comM leuB trpC2 ald-1 & Tfm (QB936: 1A708DNA, Ara\textsuperscript{+} Com\textsuperscript{+}) \\
KUS14 & com31 leuB trpC2 ald-1 & Tfm (QB936: 1A710DNA, Ara\textsuperscript{+} Com\textsuperscript{+}) \\
AY1 & cysA14 metC3 tre-12 hisH arg-1 & Akamatsu and Taguchi\textsuperscript{14} \\
HH1 & cysA14 metC3 tre-12 trpC2 leuB8 arg-1 & Tfm (AY1: QB936 DNA, His\textsuperscript{+} Leu\textsuperscript{-}) \\
HH2 & cysA14 metC3 tre-12 trpC2 leuB8 arg-1 & Tfm (AY1: HH1 DNA, His\textsuperscript{+} Leu\textsuperscript{-}) \\
HH4 & cysA14 metC3 tre-12 hisH leuB8 arg-1 & Tfm (HH2: AY1 DNA, Trp\textsuperscript{-} His\textsuperscript{-}) \\
AYG1 & cysA14 metC3 tre-12 trpC2 aroG932 arg-1 & Tfm (AY1: QB936 DNA, His\textsuperscript{+} Trp\textsuperscript{-} Ara\textsuperscript{-}) \\
AYG2 & cysA14 metC3 tre-12 trpC2 aroG932 leuB8 arg-1 & Tfm (HH4: AYG1 DNA, His\textsuperscript{+} Trp\textsuperscript{-} Ara\textsuperscript{-}) \\
5FB1 & lys-1 trpC2 & Akamatsu and Taguchi\textsuperscript{14} \\
AYK1 & cysA14 tre-12 hisH arg-1 lys-1 & Tfm (AY1: 5FB1 DNA, Met\textsuperscript{+} Lys\textsuperscript{-}) \\
AYK2 & cysA14 tre-12 hisH arg-1 lys-1 & Tfm (AY1: AYK1 DNA, Met\textsuperscript{+} Lys\textsuperscript{-}) \\
plasmid & & in this study \\
\textit{pCB1} & \textit{Cm-r Ap-r} & \textit{Cm-r Ap-r} \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Tfm: transformation; Tfm (168S: AC327 DNA, Trp\textsuperscript{+} Ade\textsuperscript{-} Str-s) indicates a Trp\textsuperscript{+} Ade\textsuperscript{-} Str-s transformant of 168S by AC327 DNA.

\textsuperscript{b} Bacillus Genetic Stock Center strain
37°C for 90 min. Then the cells were plated out onto LB agar medium with 10 µg per ml of chloramphenicol, incubated overnight at 37°C, and observed for transformation.

**Results and Discussion**

**Incorporation of the whole chromosomal DNA in protoplast lysates into competent cells of Bacillus subtilis**

Cotransfer of markers separated by a distance corresponding to 2.3% of the total B. subtilis chromosome has been previously reported by LP transformation. Linkage of markers (leuB and trpC) separated by a distance corresponding to 12% (517 kb) of the total chromosome (Fig. 1) was also found by examining the transformation of B. subtilis AC873 (leuB, trpC, ald-I) with DNA in protoplast lysates of B. subtilis AC819 (hisH, tet-1, rpmL, smo-1). When Trp', Leu', or Trp' Leu' transformants were selected, the cotransfer ratio of trpC to leuB was constant at 4.1-4.8% (Trp' Leu'/Leu') or 4.5-4.8% (Trp' Leu'/Trp') at protoplast concentrations of 2.7 x 10^6-2.7 x 10^4 per ml. The number of competent cells per ml was at least 1.6 x 10^10 cells per ml. When two markers (leuB and trpC) are separately taken up into a cell as different DNA fragments, the cotransfer ratio will rapidly fall with the decreasing donor DNA concentration. The observed constant cotransfer ratio, therefore, indicates that the two markers (separated by 517 kb) must be contiguous on a large transforming DNA. Further linkage analysis was done by examining the linkage relationship between purB and trpC. The distance of the two markers is 1,673 kb, corresponding to 40% of the total chromosome (Fig. 1). Competent cells of B. subtilis AC870 (purB, leuB, trpC, ald-I) were transformed for Ade', Trp', or Ade' Trp' with DNA of B. subtilis AC819 (hisH, tet-1, rpmL, smo-1). Figure 2 shows the number of transformants per ml as a function of the number of protoplasts per ml, namely donor DNA concentration. At low protoplast concentrations, the number of transformants per ml is directly proportional to the number of protoplasts per ml. At high concentrations, saturation of the number of transformants per ml occurs slightly. A cotransfer ratio of purB to trpC, was constant at 7-9% (Ade^- Trp'/Trp') or 3% (Ade' Trp'/Ade') at all protoplast concentrations (Fig. 2). There were at least 3.2 x 10^10 competent cells in one ml of cell culture (Fig. 2 legend). As one protoplast contains one to two DNA molecules, 2.7 x 10^5 protoplasts correspond to 2.7 x 10^7-5.4 x 10^7 DNA molecules, which are 60-120 times lower than the number of competent cells (3.2 x 10^10). Hence chromosomal

![Fig. 1. Genetic Map of the B. subtilis Chromosome. The size of the B. subtilis genome is 4,214,841 base. A degree, therefore, corresponds to about 11.7 kb. Number in the circle indicates map position of the B. subtilis chromosome. The distance of origin to cysA, rpmL, purB, metC, hisH, trpC, lys-1, leuB, arg-1, aroG, or purA is 113 kb, 130 kb, 700 kb, 1,384 kb, 2,370 kb, 2,370 kb, 2,437 kb, 2,891 kb, 3,012 kb, 3,045 kb, or 4,155 kb, respectively.](image-url)

![Fig. 2. Linkage Between purB and trpC Markers by Transformation of B. subtilis AC870 with DNA in Protoplast Lysates of B. subtilis AC819. Ade', Trp', and Ade' Trp' transformants of B. subtilis AC870 with DNA in protoplast lysates of B. subtilis AC819 were selected. The cotransformation ratio is the percent ratio of number of Ade' Trp' transformants to number of Ade' or Trp' transformants. At least 3.2 x 10^7 competent cells were in one ml of cell culture, because 3.2 x 10^7 Ade' transformants were obtained with DNA from 2.7 x 10^5 protoplasts. Symbol: ○, Number of Ade' transformants per ml; △, number of Trp' transformants per ml; ▼, number of Ade' Trp' transformants per ml; ●, cotransformation ratio of Ade' Trp' to Ade'; ▲, cotransformation ratio of Ade' Trp' to Trp'.](image-url)
DNA of *B. subtilis* must be totally incorporated into a competent cell at a time, even though there are 20 to 50 purified DNA uptake sites per competent cell. When a lower concentration of DNA is used for transformation (0.05 μg of DNA per ml culture), fewer DNA receptor sites (~13) are known to be capable of attaching DNA molecules. Even this DNA concentration is rather higher than those of our experimental conditions (0.013 μg - 0.013 ng of DNA per ml culture).

Similar results were obtained with the cotransfer ratio of purA to trpC, which are separated by 1,782 kb corresponding to 42% of the total chromosome (Fig. 3). All of the observations indicate that the whole chromosomal DNA of *B. subtilis* is incorporated into a competent cell.

**Linkage analysis by DNA in protoplast lysates of *B. subtilis***

Figure 4 shows the linkage analysis for DNA in protoplast lysates of *B. subtilis*. Linkages of hisH to trpC (3.6 kb), cysA to rpsL (17 kb), and of lys-1 to trpC (63 kb) were examined using *B. subtilis* AYG2 and *B. subtilis* 168S as a recipient and a donor, respectively. Linkages of arg-1 to aroG (34 kb), of arg-1 to leuB (121 kb), of arg-1 to cysA (1,316 kb), of arg-1 to rpsL (1,333 kb), and of arg-1 to metC (1,628 kb) were examined using *B. subtilis* AYG2 and *B. subtilis* 168S as a recipient and a donor. Cotransfer ratios decreased with increasing marker distances of the chromosome up to 121 kb and showed a plateau from 121 kb to 1,628 kb (Fig. 4). The results indicate that the cotransformation can occur with markers separated by at least 121 kb. The cotransfer ratios of markers separated over 121 kb indicate coaggregation (double transformation) by either fragmentation of the incorporated whole chromosomal DNA or a limited extent of DNA exchange, done by helicases in wild-type strain. The latter case partly contributes to the transformation, because cotransfer ratios of a mutant (ist-1 mutant showing high frequency of interspecific transformation and high cotransformation) are significantly higher (~two times) than those of wild-type strain (Akamatsu, T. et al., unpublished results).

**Involvement of several Com proteins on the transformation**

![Fig. 3. Linkage Between purA and trpC Markers by Transformation of B. subtilis AC871 with DNA in Protoplast Lysates of B. subtilis AC819.](image)

![Fig. 4. Linkage Analysis by Chromosomal DNA in Protoplast Lysates of B. subtilis.](image)
The transforming DNA molecules have been thought to attach by their ends to the cells only at DNA uptake sites.\(^2\) Then only one transforming DNA molecule at a time is taken up by the cell.\(^2,3\) The average number of nucleotide lengths of DNA entering the cell per second has been calculated to be 138 bases at 37°C.\(^3\) The length of the total chromosomal DNA is 4,214,841 bases.\(^2\) As the DNA is incorporated within 30 min under our experimental conditions, at least 2,340 bases of DNA must be taken up per second, assuming that the DNA in protoplast lysates are also attached to the competent cells, taken up in a linear fashion, and fragmented as just described for isolated DNA.\(^1\) The difference of the two uptake rates is significant (about 20 times). Hence we examined whether several Com proteins are also required for the transformation with DNA in protoplast lysates or not. Involvement of ComK was examined using isolated DNA or DNA in protoplast lysates. The transformation frequency of the comK strain with DNA in protoplast lysates was 300 times lower than that of a wild-type strain (Table 2). The result is quite similar to that using isolated DNA (Table 2). Transformation frequency of the comK strain with isolated plasmid DNA is also lower than that of the wild-type strain (Table 2). Similar results were obtained for all of the com strains examined (com-9, comM, com-31, com-14 and comR strains) (Table 2). The ComK protein can induce the transcription of recA as well as comK itself and all of the late competence operons (the comC gene and the comE, comF, and comG operons) that are essential for the uptake of exogenous DNA in macromolecular form.\(^4,5\) Hence proteins taking up the isolated DNA must be also involved in uptake of DNA in protoplast lysates.

Using DNA made available by gentle lysis of a stable L-form, up to 25% of the genome of \textit{B. subtilis} is taken up.\(^6\) It is known that after the gentle lysis of \textit{B. subtilis} protoplasts, the chromosome remains intact and attached to the cell membrane.\(^7\) We postulate incorporation of the whole chromosomal DNA into competent cells from the high cotransfer observed using DNA in protoplast lysates. The data in Fig. 2 and Fig. 3 conclusively prove our hypothesis.

At least 2,340 bases of DNA must be taken up per second at 37°C, assuming that the transformation pathway is similar to that by isolated DNA.\(^1\) As only 138 bases of isolated DNA is taken up per second, difference of the two rates is 20 times. In addition to the well-known Com proteins, accessory proteins or certain structures of DNA receptor sites are likely required for the transformation with DNA in protoplast lysates. The membrane structure called trans-

### Table 2. Involvement of Com Proteins in High Frequency of Transformation of \textit{B. subtilis}

<table>
<thead>
<tr>
<th>Recipient</th>
<th>(Relevant genotype)</th>
<th>Donor</th>
<th>Selection</th>
<th>Ratio* (Number of transformants per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QB936</td>
<td>wild-type</td>
<td>Protoplast lysates(^b)</td>
<td>Leu(^+)</td>
<td>1.0 ((2.5 \times 10^6))</td>
</tr>
<tr>
<td>KUS13</td>
<td>comK</td>
<td>Protoplast lysates</td>
<td>Leu(^+)</td>
<td>3.2 (\times 10^{-2})</td>
</tr>
<tr>
<td>KUS12</td>
<td>com-9</td>
<td>Protoplast lysates</td>
<td>Leu(^+)</td>
<td>5.9 (\times 10^{-2})</td>
</tr>
<tr>
<td>KUS14</td>
<td>comM</td>
<td>Protoplast lysates</td>
<td>Leu(^+)</td>
<td>4.7 (\times 10^{-2})</td>
</tr>
<tr>
<td>KUS15</td>
<td>com31</td>
<td>Protoplast lysates</td>
<td>Leu(^+)</td>
<td>5.0 (\times 10^{-2})</td>
</tr>
<tr>
<td>QB936</td>
<td>wild-type</td>
<td>Protoplast lysates</td>
<td>Aro(^+)</td>
<td>1.0 ((3.5 \times 10^6))</td>
</tr>
<tr>
<td>KUS10</td>
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<td>Protoplast lysates</td>
<td>Aro(^+)</td>
<td>2.2 (\times 10^{-2})</td>
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<tr>
<td>KUS11</td>
<td>comR</td>
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<td>Aro(^+)</td>
<td>2.4 (\times 10^{-2})</td>
</tr>
<tr>
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<td>Chr. DNA(^d)</td>
<td>Leu(^+)</td>
<td>1.0 ((3.7 \times 10^5))</td>
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<tr>
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<td>comK</td>
<td>Chr. DNA</td>
<td>Leu(^+)</td>
<td>8.3 (\times 10^{-4})</td>
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<tr>
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<td>4.6 (\times 10^{-4})</td>
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<td>Leu(^+)</td>
<td>6.9 (\times 10^{-4})</td>
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<td>Chr. DNA</td>
<td>Aro(^+)</td>
<td>1.0 ((3.8 \times 10^5))</td>
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<td>com14</td>
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<td>Aro(^+)</td>
<td>5.4 (\times 10^{-4})</td>
</tr>
<tr>
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<tr>
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<td>pCBI DNA</td>
<td>Cm-r</td>
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<td>pCBI DNA</td>
<td>Cm-r</td>
<td>&lt;3.6 (\times 10^{-3})</td>
</tr>
<tr>
<td>KUS11</td>
<td>comR</td>
<td>pCBI DNA</td>
<td>Cm-r</td>
<td>&lt;2.6 (\times 10^{-3})</td>
</tr>
</tbody>
</table>

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* Ratio is calculated by dividing the number of transformants of mutant strain by the number of transformants of wild-type strain.

\(^b\) A portion (0.1 ml) of \textit{B. subtilis} 168S protoplast suspension containing 2.7 \(\times 10^9\) protoplasts (about 0.013 μg DNA) was added to 1 ml of competent cell culture.

\(^d\) Ten mg of purified chromosomal DNA of \textit{B. subtilis} 168S or purified plasmid DNA was added to 1 ml of competent cell culture.
formsome in *Haemophilus influenzae* or *Haemophilus parainfluenzae* is one of the attractive structures for the following reasons; (1) it protrudes outside the competent cell, (2) it occurs at the same time as the appearance of new proteins in the cell envelopes, and (3) it is considered to be a means of increasing contact between the exterior of the cell and its inside (particularly the chromosome).28,29

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Total Uptake of the *B. subtilis* Genome by Competent Cells


