Molecular analysis of the bacillus subtilis transformation process

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CHAPTER VIII

SUMMARY AND GENERAL CONCLUSIONS

Transformation of *Bacillus subtilis* presents an interesting model system for the study of DNA uptake and recombination in gram-positive bacteria. This dissertation deals with several aspects of these fundamental processes. After a brief introduction into the relevant literature of transformation and the use of plasmids in *B. subtilis* (Chapter I), experiments are described aimed at 1) the elucidation of the mechanism of uptake and processing of plasmid DNA (Chapters II-IV) and 2)the identification and functional analysis of proteins involved in recombination in *B.subtilis* (Chapters V-VII).

The introduction of useful plasmids into *B. subtilis* greatly enlarged the possibilities of molecular cloning in this host. This application made it of particular interest to study the transformation process in *B. subtilis* with plasmid as model DNA. Although competent cells are readily transformed with plasmid DNA, it appeared however that, in contrast to monomers, only multimeric plasmid DNA was biologically active. However, in an alternative transformation system, making use of polyethylene glycol treated protoplasts of *B. subtilis*, all plasmid forms, including monomers, were biologically active and transformed with high efficiency. These findings stimulated the research concerning the mechanism of uptake and processing of different forms of plasmid DNA in these two transformation systems, as is descibed in the first part of this thesis.

Chapter II describes the investigation of the physical fate of plasmid DNA during transformation of competent cells. All plasmid DNA appears to be present intracellularly as single-strands of DNA, irrespective of the

molecular structure of the donor plasmid. Whereas single-strands of monomeric plasmid DNA become finally degraded, multimeric plasmid DNA results in the intracellular formation of (partially) double-stranded DNA. Based on these results a model for the biological activity of multimeric DNA is proposed, which takes into account single-stranded entry and intracellular reannealing of complementary single strands derived from one multimeric molecule.

To examine whether the conclusions reached with respect to the uptake and processing of plasmid DNA in competent cells were not limited to the major fraction of plasmid DNA, but also applied to the few biologically active plasmid molecules, plasmid transformation was also studied in transformation-deficient mutants (Chapter III). The results strongly suggest that all plasmid DNA, including the biologically active molecules, enter competent cells in the same way, i.e. in the single-stranded form. With the aid of this additional information a general model for the plasmid transformation in *B. subtilis* competent cells is proposed, which accounts for the biological activity of plasmids containing various kinds of repeats. The inefficiency of plasmid transformation using competent cells is interpreted in terms of probability to survive intracellular degradation before complementary single strands can reanneal and, after DNA synthesis, can circularize.

By using physico-chemical methods in combination with the analysis of mutants impaired in plasmid transformation when grown to competence, the uptake and processing of plasmid DNA in polyethylene glycol treated protoplasts was studied (Chapter IV). After entry into the protoplasts, which is not reduced in mutants impaired in DNA entry as competent cells, all plasmid DNA is present as double-stranded,undamaged,molecules. Quantitive analysis showed that almost each plasmid molecule which enters gi-

ves rise to a transformant, explaining the high efficiency of plasmid transformation in this system.

In relation to the differences in DNA uptake and processing in the two plasmid transformation systems, the question may be posed which of these is the most suitable for molecular cloning. Several genes have been cloned using *B. subtilis* competent cells. However, relatively high DNA concentrations are required during ligation to favour the formation of internal repeats which are requisite to endow the ligation products with biological activity. Since the processing of such repeated plasmids may lead to the formation of deletions, it is recommended to introduce primary ligation products into *B. subtilis* by protoplast transformation. In this way advantage is made of the undamaged entry and hence of the high transformation frequencies in protoplasts, as is practised in the cloning experiments described in Chapter VII.

To complement existing information on recombination during transformation of *B. subtilis* competent cells, proteins involved in recombination were studied as is described in the second part of this thesis.

By comparing proteins present in wildtype strains and isogenic recombination-deficient mutants with the aid of two-dimensional gelelectrophoresis, a 45 kD recombination protein was identified, which is induced during competence development and by treatments which damage DNA or inhibit DNA replication (Chapter V). This protein is absent in several *rec*-mutants which are all impaired in the formation of an unstable or stable donor-recipient complex during transformation (Chapter VI). Therefore, it is inferred that the 45 kD recombination protein participates in the early steps of the formation of this complex.

One mutant, carrying the temperature-sensitive *rec*E45 mutation, contains a 45 kD protein with an increased isoelectric point (Chapter VI).

After limited proteolysis, the wildtype and mutant protein yielded similar fragments, indicating that the *rec*E gene specifies the 45 kD recombination protein. Since the mutant protein was not inducible by treatments which induced the wildtype recE protein, it is assumed that the recE protein affects its own synthesis. On the basis of these findings a regulation model is proposed which postulates that the recE protein is capable of inactivating a repressor of the *rec*E gene.

Both the proposed mode of regulation and the function of the *B. subtilis* recE protein bear a great deal of similarity with that of the well studied *Escherichia coli* recA protein. In order to provide experimental evidence for the possible functional equivalence of these two proteins, DNA fragments containing the entire *E. coli rec*A gene were cloned in a *B. subtilis* expression plasmid (Chapter VII). When the *rec*A gene is under control of the vector located promoter, the *E. coli* recombination gene is expressed and the recA protein is detectable in minicells and whole cells. This type of analysis further showed that - like the recE protein - the recA protein is also inducible in *B. subtilis*. This supports the previous hypothesis that the synthesis of the recE protein is similarly regulated as that of the *E. coli* recA protein.

All *B. subtilis* mutants which lack the recE protein and hence are impaired in the formation of a complex between single-stranded donor DNA and the homologous recipient DNA, are complemented by the *E. coli rec*A gene expressing plasmid (Chapter VII). Since the recA protein promotes the assimilation of single-stranded DNA into homologous duplex DNA *in vitro* (resulting in the formation of a D loop), the most simple interpretation of the current results is that the regulation of recombination in *B. subtilis* transformation occurs in a similar way. This is vizualized in Figure 1.

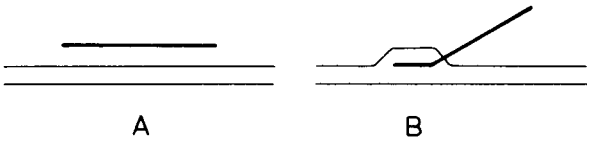

Figure 1. Model of initiation of recombination during transformation in *B. subtilis*. The bold line represents single-stranded donor DNA, unpaired in *A*. Pairing with the complementary strand of the recipient duplex DNA in *B* results in the formation of a D loop. Though depicted, for the sake of simplicity, as two straight lines, the recipient DNA may be in the superhelical form.

Owing to the cloning and expression of the *E. coli recA* gene in *B. subtilis*, an experimental system is now available which can be used to test a number of biochemical properties of the recA protein *in vivo* with the use of the well characterized *B. subtilis* transformation system.