university of groningen

University Medical Center Groningen

University of Groningen

Characterization of plasmids for gene cloning in bacilli af high temperatures

Oskam, Linda

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1991

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Oskam, L. (1991). *Characterization of plasmids for gene cloning in bacilli af high temperatures*. s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 27-09-2023

SUMMARY

The bacterial genus *Bacillus* continues to be a focus of intensive physiological, biochemical and genetical research. The various members of the genus are capable of growth in a wide range of pH and temperature conditions and produce a number of industrially valuable enzymes. The recent development of a number of stable and efficient host-vector systems for bacilli has opened the opportunity to produce large quantities of such enzymes. In particular, *B.subtilis* has become the paradigm for research in bacilli and other gram-positive bacteria, since the genetics, biochemistry and physiology of this organism have been well-characterized.
Recent years have seen an increased interest in physiological processes in bacilli, that occur at higher temperatures. For biotechnological application such processes have several potential advantages, such as higher rates of product formation, decreased energy expenditure both for agitation (due to the lower viscosity of the culture broth) and for cooling during vigorous fermentation, and a reduced danger of contamination by pathogenic organisms. The genus *Bacillus* comprizes a number of thermophilic representatives which may be suitable for the use in biotechnological processes at higher temperatures. However, one important requirement should be met: the availability of stable and efficient host-vector systems for these organisms.
Despite the considerable interest of biotechnology for thermophilic bacilli, the research in the field of plasmid biology of this group of organisms is still in its infancy. pTB19 is a plasmid originally isolated from a thermophilic *Bacillus*. The plasmid is relatively large (26.5 kilo basepairs [kb]), has a low copy number and encodes resistance against the antibiotics kanamycin and tetracycline. Upon selection on kanamycin the integrated plasmid pTB913 is liberated from pTB19. This plasmid is much smaller than the parental plasmid (4.5 kb) and has a higher copy number.
This thesis deals with the characterization of plasmids pTB19 and pTb913. Chapters II and III describe plasmid pTB913. In Chapter IV, a second plasmid integrated into pTB19 is described that is strongly related to pTB913. Finally in Chapter V, studies are described aimed at the development of cloning vectors for use at higher temperatures in *B.subtilis* and the thermophile *Bacillus stearothermophilus* on the basis of (parts of) pTB19.

Chapter I provides a survey of the current state of knowledge of several aspects of plasmid biology of *Bacillus*. The emphasis is on replication mechanisms, plasmid stability and plasmid biology of thermophilic bacilli.

In Chapter II the sequencing to completion of plasmids pTB913 and pMV158 is described. Both plasmids appeared to replicate according to the rolling-circle model and contained a *palU* minus origin of replication homologous to that of the *Staphylococcus aureus* plasmid pUB110. In addition, pMV158 contained a *palA* minus origin. In both plasmids deletion of the *palU* minus origin led to the accumulation of single-stranded DNA replication intermediates. The *palU* minus origin of pTB913 was flanked on one side with an open reading frame specifying bleomycin resistance. On the other side of the *palU* minus origin pUB110, pTB913 and pMV158 contained a palindromic structure (the RS_A-site) followed by an open reading frame. Comparison of these RS_A-sites with those of plasmids pT181, pE194, pNE131 and pT48 indicated the existence of two related groups of RS_A-sites, the former three specifying the first group, the latter four the second group. Comparison of the open reading frames flanking the RS_A-sites in pTB913, pMV158, pUB110, pT181 and pE194 revealed that the deduced amino acid sequences of the Pre or Mob proteins (Pre designates plasmid recombination enzyme; Mob mobilization) were highly related, especially in their N-terminal parts. In all probability, the Pre/Mob proteins use the RS_A-site as the target in Rec-independent recombination or in conjugative mobilization. Two groups of Pre/Mob proteins could be distinguished: the first three Pre/Mob proteins mentioned above belonged to one group and the latter two to the other. The observation that a particular class of RS_A-sites was accompanied by a particular class of Pre/Mob proteins

suggests that the interaction between the Pre/Mob proteins and their RS_A-sites is highly specific.

Chapter III describes the structure of pTB913 when integrated in the parental plasmid pTB19. DNA sequence analysis revealed that the integration had resulted, by an unknown mechanism, in the formation of a 55 basepairs direct repeat. Integration had taken place within the coding sequence of the replication initiation gene of pTB913, rendering the integrated copy of the plasmid unable to drive plasmid replication. It was shown that the formation of pTB913 from pTB19 was a very rare event. However, once pTB913 was formed, *B.subtilis* cells containing pTB913 appeared to have a growth advantage over cells containing pTB19, especially upon selection at high concentrations of kanamycin. This offered a satisfactory explanation for the accumulation of cells containing pTB913.

In Chapter IV the nucleotide sequence of the pTB19 region conferring resistance to tetracycline is described. In addition to the rolling-circle plasmid pTB913, nearly the whole sequence of a second rolling-circle plasmid, carrying the tettracycline resistance determinant, appeared to be present in pTB19. The tetracycline resistance gene was nearly identical to the corresponding genes of a number of other rolling-circle plasmids. Moreover, this region contained a *palU* minus origin identical to those of pUB110 and pTB913. As in pUB110, pTB913 and pMV158, an RS_A-site and a gene encoding a mobilization protein were present next to the minus origin. The RS_A-site differed in only one basepair from the RS_A-site of pUB110 and in two basepairs from that of pTB913. The encoded mobilization protein appeared to be a hybrid between the mobilization proteins encoded by pUB110 (N-terminal part) and pTB913 (C-terminal part).
The results of experiments in which the RS_A-site and/or the *mob* gene of pUB110 were exchanged for the corresponding regions from the tetracycline resistant part of pTB19, indicated that the hybrid *mob* gene enabled mobilization at the same frequency as that of pUB110. However, the altered RS_A-site led to a drastic reduction in the mobilization frequency. This lends strong support to the idea put forward in Chapter II that the RS_A-site is the target site for Mob protein and is involved in the process of conjugative mobilization.
Upstream of the tetracycline resistance gene the 3' end (275 basepairs) of a replication initiation gene, homologous to that of pUB110, was located, whereas the 5' end (250 basepairs) of this gene was located downstream of the *mob* gene. The remaining 492 basepairs of the central part of the replication initiation gene were absent. As with pTB913, integration of the tetracycline resistance-conferring rolling-circle plasmid in pTB19 had taken place within the replication initiation gene, also rendering this integrated plasmid incapable of autonomous replication.

The results described in Chapters III and IV indicate that pTB19 was a cointegrate consisting of at least three replicons: (i) the rolling-circle plasmid pTB913; (ii) the tetracycline resistance-conferring plasmid that also belonged to the family of rolling-circle plasmids; and (iii) a plasmid based on the RepA determinant replicating via a theta mechanism. It was hypothesized that, as a result of mobilization, the rolling-circle plasmids had been transferred to a thermophilic *Bacillus* containing the RepA replicon. Such plasmids may well be incapable of self-maintenance under these adverse conditions (an example of this is described in Chapter V). It is speculated that only plasmids that become integrated into the RepA replicon could survive under these unfavourable conditions. Probably, in this system strong selection exists in favour of plasmids in which the replication initiation genes of the integrated rolling-circle plasmids are interrupted. The non-functionality of the replication initiation gene is expected to prevent structural plasmid instability mediated by the presence of large direct repeats and multiple replication origins of the related integrated rolling-circle plasmids.

In Chapter V studies on the development of stable host-vector systems at higher temperatures are described. To this purpose,

plasmid pTB913, described in Chapters II and III, was used. The segregational stabilities of pTB913-derivatives in *B.subtilis* at 37°C and 47°C and in *B.stearothermophilus* at 57°C were analyzed. In *B.subtilis* at 37°C the stability of the plasmid appeared to be reduced when the minus origin was absent and when the plasmid was enlarged through the insertion of random DNA fragments from *Escherichia coli*. This seemed to result from a reduction in the copy number of the plasmid, presumably due to the accumulation of single-stranded and high-molecular weight replication products. At 47°C the copy numbers were twice as high as those observed at 37°C, which might explain the full segregationally stability of all six plasmids in *B.subtilis* at 47°C.

In order to develop host-vector systems for use at temperatures above 47°C, pTB913-derivatives were transferred to two strains of *B.stearothermophilus*. The segregational stabilities appeared to be strongly strain-dependent. In strain CU21 a high degree of segregational instability was observed with pTB913. This seemed to result from the production of large amounts of single-stranded DNA replication intermediates due to a low activity of the minus origin. In contrast, in strain NUB3621 pTB913 was relatively stably maintained. In this strain the minus origin seemed to be active, as follows from the observation that single-stranded DNA was efficiently converted to double-stranded plasmid DNA. This is likely to be an important reason for the observed higher stability of pTB913 in strain NUB3621 compared to strain CU21. It was concluded that pTB913 was a suitable vector for molecular cloning at elevated temperatures in certain *Bacillus* strains, including *B.stearothermophilus* NUB3621.

Experiments with a plasmid based on the RepA determinant, conferring theta replication, in NUB3621 indicated that this plasmid was lost very rapidly from the cell population and was, therefore, less suitable as a cloning vector in this strain.