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Replication control of the *Staphylococcus aureus* chloramphenicol resistance plasmids pC223 and pUB112 in *Bacillus subtilis*

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ABSTRACT

A detailed physical and functional map of the chloramphenicol (Cm) resistance plasmid pC223 from Staphylococcus aureus was compiled. The plasmid's basic replicon and origin of replication were located and their nucleotide sequences determined. Two small RNAs of 92 and 155 nt, demonstrated by in vitro transcription with vegetative Bacillus subtilis RNA polymerase, were depicted as copy number regulating (cop) and incompatibility (inc) functions in Bacillus subtilis. pC223 and pUB112, another S.aureus Cm resistance plasmid, which exhibits marked sequence homology with pC223 and codes also for two small copRNAs, could be classified as members of the pT181-plasmid family (1). Copy numbers and segregational instability of pC223, pUB112 and deletion derivatives of both in B.subtilis showed great differences despite of their homologous basic replicons.

INTRODUCTION

Since the discovery by Ehrlich (2) that staphylococcal antibiotic resistance plasmids can be replicated and their genetic information be expressed in Bacillus subtilis, great progress has been made in using them as cloning vectors in the foreign host. However, important factors for their maintenance like copy number control, incompatibility functions and segregational stability have been studied intensively in Staphylococcus aureus, but hardly in B.subtilis, in which most of them are not stably inherited indicating that critical elements in host-plasmid interaction are dysfunctional (1).

The tetracyclin (Tc) resistance plasmid pT181 is the well-studied prototype of a family of small S.aureus plasmids, which consists of different incompatibility groups (1,3). These plasmids control their copy number by inhibitory countertranscripts (copRNAs) which interact with the untranslated leader of the mRNA of an essential replication initiation protein (Rep) thus inhibiting rep mRNA translation (1,4). pT181 possesses two rep mRNAs, starting at different positions, and two different copRNAs, initiating at the same point (4). Members of the pT181-family are the Cm resistance plasmid pC221 (5)

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and the streptomycin (Sm) resistance plasmid pS194 (6), which show great homology in their rep genes (including the leaders) and in the structures of the replication origin (ori) with pT181 (7). The Rep protein of the pT181-like plasmids initiates plasmid replication by nicking at a specific sequence in the ori region, that is situated in the 5'-region of the Rep-coding sequence (7,8). Different from the pT181-family are two groups of grampositive plasmids, the pUB110- and the pSN2- families, each containing plasmids from B.subtilis and S.aureus, that belong to a single incompatibility group (1). They vary from the pT181-group in copy number control as well as in their rep and ori sequences (9,10,11).

The S.aureus Cm resistance plasmids pC223 (12) and pUB112 (13,14) have not been studied yet in regard to copy number regulation. In the present study, the functional map and the nucleotide sequence of the basic replicon (the smallest piece of a plasmid that is still able to replicate, 15) of pC223 have been determined and copy number control and segregational stability of pC223 and pUB112 in B.subtilis have been studied. Copy numbers and segregation rates of both wild-type plasmids and of deletion derivatives and copy number mutants in B.subtilis have been compared to those of five different S.aureus plasmids. The copy numbers in B.subtilis varied remarkably, even for plasmids of the same family, and an instable inheritance of most staphylococcal plasmids in the foreign host was confirmed.

MATERIAL AND METHODS

Bacterial strains and plasmids

The S.aureus plasmids pC223 (12, obtained by R.Novick, New York, in S.aureus strain RN154) and pUB112 (13,14), pC221 (5), pUB110 (13), (all obtained by R. Brückner, Heidelberg), pC194 and pE194 (6, obtained by E. Zyprian and J. Tennigkeit, Heidelberg), pRB311 (the circularized large BclI fragment of pUB112 (14), obtained by R. Brückner, Heidelberg) and the pC221 deletion plasmid pCW41 (16, obtained by W.V. Shaw, Leicester, in S.aureus strain 8325-4 recA1 his7) were transformed and maintained in B.subtilis strain BD 170 trp2 thr5 (17). The construction of the pC223 deletion derivatives pMEH25, pMET9, pMER8 and pMET3 is described in this work.

Media and growth conditions

B.subtilis and S.aureus were grown in trypticase soy broth (TSB) medium (BBL, Cockeysville) and plated on LB agar plates. Plasmid-containing cells were grown in TSB with 20 µg of the appropriate antibiotic / ml.

Plasmid DNA manipulations and transformation

Large-scale plasmid preparations from B.subtilis were done according to

Birnboim and Doly (18) and from S.aureus by a combination of methods described by Novick (12), Holmes and Quigley (19) and Schaberg et al. (20), including the purification of plasmid DNA by CsCl density gradient centrifugation. Mini plasmid preparations were carried out by the same methods with slight modifications. Restriction, Bal 31 digestion, ligation and phosphorylation of DNA were done under conditions recommended by the suppliers and followed standard procedures (21). Competent B.subtilis BD 170 cells were prepared according to Contente and Dubnau (22). Analysis and isolation of DNA restriction fragments were performed by agarose and polyacrylamide gel electrophoresis (21).

Incompatibility assays

Unilateral incompatibility was scored by displacement tests (23). The displacing plasmid was transformed into B.subtilis BD 170 containing the plasmid, that was to be displaced, under selection for both plasmids. The heteroplasmid strain was grown in successive overnight cultures under non-selective conditions and mini plasmid preparations from each culture were done. Incompatibility was scored by the disappearance of the displaced plasmid.

Segregational incompatibility was examined by reciprocal intrapool variation tests (24). The reciprocal variation of the copy numbers of two different plasmids in the same B.subtilis strain, relative to the selective conditions under which the culture was grown, was taken as evidence for segregational incompatibility.

DNA sequencing

For DNA sequencing the dideoxy chain termination method (25) with covalently closed plasmid DNA as template was used. Restriction fragments of pC223 and pUB112 were cloned into Escherichia coli vector plasmids pBR322 (26), pRB273 (14), pUR250 (27) or pUC18 (28). As primers were used a 16-mer primer, complementary to sequences adjacent to the Eco RI-site of pBR322 and pRB273 (New England Biolabs) or the 16-mer pro 42 and 17-mer pro 43 primers, complementary to sequences adjacent to the polylinker sequences of pUR250 and pUC18 (Progen, Heidelberg).

Sequencing reactions were analyzed on 6% or 8% polyacrylamide/8 M urea gels.

In vitro transcription

Transcription assays were carried out as described (29).

Determination of plasmid copy numbers

To determine the intracellular plasmid content of B.subtilis, 1.5 ml of an exponentially growing culture (3 x 10^8 cells/ml; A_{600} = 2) were centrifuged at

8000 rpm in a microfuge, and the sediment was treated as described by Birnboim and Doly (18), but without RNA digestion. The total amount of nucleic acids recovered in the mini-plasmid preparation after the salt precipitation step was determined by measuring A_{260}. One half of the sample was applied untreated, the other one after treatment with a linearizing restriction enzyme and RNAse to an 1% agarose gel, which was stained after electrophoresis in an aqueous solution of 1 µg ethidium bromide/ml for 30 min and destained in distilled water for 60 min (30). The gel was illuminated with u.v. light and photographed. The amount of plasmid DNA in the mini-plasmid preparation was quantitated with respect to the concentration of total nucleic acids, consisting of RNA, plasmid DNA and some residual chromosomal DNA, by microdensitometry of film negatives, taken of the gel, with a Joyce-Loebl 3 CS densitometer, and integrating the corresponding peaks (31). All determinations were carried out at least three times.

Segregational instability of plasmids

Freshly plasmid-transformed B.subtilis colonies were replica plated on non-selective agar plates. The new colonies were tested for plasmid content by replica plating on agar plates with 20 µg of the appropriate antibiotic/ml and were again relpica plated on non-selective plates. The colonies from the non-selective plates were replica plated just as described and so on. The segregation rate per cell and generation was calculated according to Iordanescu (32).

RESULTS

Physical and functional analysis of pC223

After having compiled a detailed restriction map of the 4.6 kb plasmid pC223 (Fig.1) the location of the cat gene and the direction of its transcription were determined by cloning restriction fragments in the E.coli vector pBR322, by analysing pC223 deletion derivatives in B.subtilis and finally by DNA sequencing (33). The following deletion plasmids, still capable of replicating in and confering Cm resistance to B.subtilis, were obtained (Fig.1): pMEH25 (3.2 kb), constructed by circularization of the ligated large and small pC223-HaeII-fragment, pMET9 (2373 bp) by partial TaqI digestion of pMEH25 and religation of four of six fragments, and pMER8 (2148 bp) by circularization of the large pMET9-RsaI-fragment.

Localization of replication and incompatibility functions of pC223

pMET3 was the smallest plasmid that could be isolated, together with larger plasmids carrying the cat gene, from Cm resistant B.subtilis colonies, obtained by transformation with religated partially TaqI-digested pMEH25-DNA.

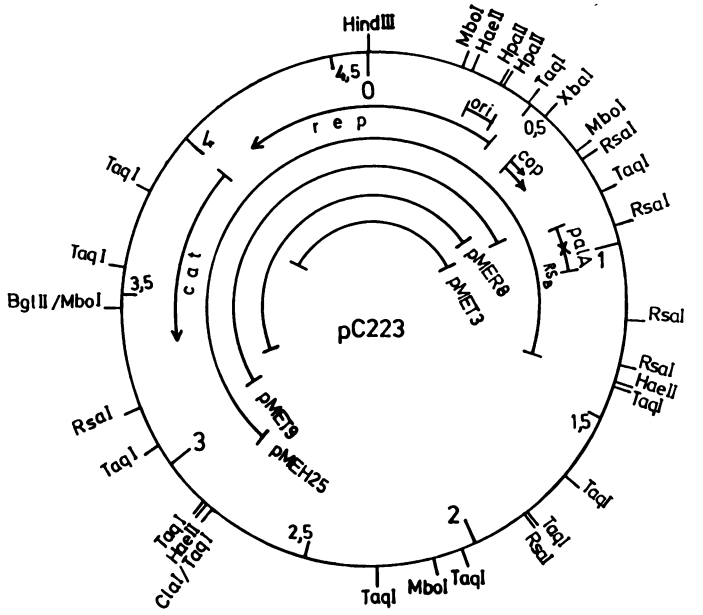


Figure 1. Restriction and functional map of pC223 and the four deletion derivatives pMEH25, pMET9, pMER8 and pMET3. The unique HindIII-site was chosen as point of reference (4.6 kb/0 kb). Map units are given in kb. Plasmid pC223 has a size of 4.6 kb, pMEH25 of 3.2 kb, pMET9 of 2373 bp and pMET3 of 1620 bp. Moreover, by DNA sequencing and restriction analysis (marked by *) one AvaII-site (pos. 1336), one Bst E II-site (pos. 3419), four DdeI-sites (pos. 445, 1425*, 1610*, 1950*), five HinfI-sites (pos. 432, 493, 3572, 3694, 3843), two HphI-sites (pos. 514, 4075) and two SphI-sites (pos. 1605*, 2130*) have been determined. Enymes AluI, DraI and MboII cleave pC223, but the sites have not been localized. Restriction endonucleases that do not cut pC223 include: AccI, AvaI, BalI, Bam H I, BclI, BglI, Bst N I, Eco R I, Hae III, Hind II, HpaI, KpnI, PstI, PvuI, PvuII, SalI, SstI, XhoI and XorII. For explanation of the indicated functions see results and discussion.

It consists of two TaqI-fragments of 1274 bp and 346 bp (Fig.1). Thus, the basic replicon of pC223 could be located between its TaqI-site at position 3817 and RsaI-site at position 723. Replicons lacking the 1274 bp TaqI-fragment could never be isolated and plasmids, missing the 346 bp TaqI-fragment, could only be scored in co-existence with plasmids, which harboured this fragment, indicating that the large TaqI-fragment carries a cis active replication function, presumably the origin of replication, and that the small one is necessary for a complementable trans active replication function, probably the pC223 replication initiation protein RepM.

The ori of a grampositive plasmid integrated into a gramnegative replicon

should promote the replication of the latter in a grampositive host when the appropriate Rep protein is provided in trans. This rationale was applied to locate the pC223 ori. The MboI-TaqI-fragment between positions 276 and 491 was cloned by means of linkers into the single XbaI-site of pUR250cat, which had been constructed by insertion of the MboI-TaqI-cat-fragment of pUB112 (14) into the E.coli vector pUR250. In contrast to pUR250cat, the new plasmid, pUR215cat, could transform B.subtilis BD 170 harbouring pMET110, a construct of pMET3 and the kanamycin (Km) resistance determinant of pUB110 (34), to Cm resistance. Apparently, the pC223 Rep protein, coded on pMET110, initiates the replication of pUR215cat in trans at the pC223 ori on the cloned fragment, as emphasized by the behaviour of both plasmids as autonomous replicons (see Fig.2). pMET110 and pUR215cat, that carry the same ori, are segregational incompatible in B.subtilis, as demonstrated by the reciprocal intrapool variation test (24). Fig.2 shows that copy numbers of the two plasmids varied reciprocally relative to the selective conditions, the copy number of pUR215cat never being higher than that of pMET110 because of the dependance of its replication in B.subtilis on the presence of pMET110.

By displacement testing an unilateral trans active incompatibility function of pC223 was located on the TaqI-fragment between positions 491 and 837. The fragment was cloned into the AccI-site of the Km resistance vector pRB103 (14), yielding plasmid pMET360. B.subtilis BD 170, harbouring pMER8, was transformed by pRB103 or by pMET360 to Cm and Km resistance. Both transformants were then grown in successive overnight cultures under non-selective conditions. Fig.3 shows plasmid preparations after one, two, three and four passages. It is apparent that pMET360 displaced pMER8, whereas pRB103 did not.

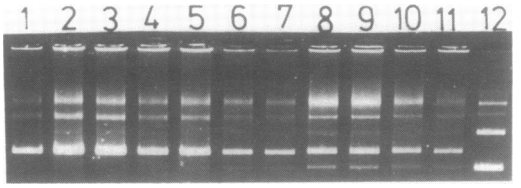


Figure 2. Reciprocal intrapool variation test. Gel elelectrophoresis of mini plasmid preparations from B.subtilis carrying plasmid pMET110 (lane 1), from E.coli carrying plasmid pUR215cat (lane 12) and from B.subtilis heteroplasmid strains, transformed by both plasmids (lanes 2-11). The selective conditions for the heteroplasmid strains varied as follows: lanes 2 and 3: 20 µg Km/ml; lanes 4 and 5: 50 µg Km/ml and 5 µg Cm/ml; lanes 6 and 7: 20 µg Km/ml and 20 µg Cm/ml; lanes 8 and 9: 5 µg Km/ml and 50 µg Cm/ml, lanes 10 and 11: 20 µg Cm/ml.

Nucleotide sequence and in vitro transcription analyses

After the essential replication and incompatibility functions of pC223 had been located, the nucleotide sequence of its basic replicon and adjacent regions was determined (Fig.4). In this sequence an open reading frame for a polypeptide of 314 amino acids was detected that, in analogy to plasmids pT181 and pC221, represents the replication protein of pC223, RepM, the ori sequence being included in the RepM-coding region. Upstream of this region a large palindromic sequence, homologous to the palA-sequences of pT181, pC221 and other S.aureus plasmids, which are necessary for proper replication in S.aureus (35), could be identified. The sequence preceding the repM gene contains two hypothetical promoter regions on the coding and one on the opposite strand. The latter is followed by terminator-like sequences t_1 and t_2 after about 100 and 150 bp. In analogy to pT181 and pC221 these signals were assumed to regulate the synthesis of repM mRNA and copRNAs.

To verify the existence of these RNAs, in vitro transcription was performed with $E\sigma^{43}$ RNA polymerase from B.subtilis using the 346 bp TaqI fragment, which had been shown to carry a trans acting incompatibility function, as template. As depicted in Fig.5, two transcripts of 155 and 92 nt were obtained. They also appeared as prominent bands when the supercoiled plasmid DNAs of pC223, of its deletion derivatives and of pMET360 which contains this TaqI fragment (see above) were transcribed (data not shown). To

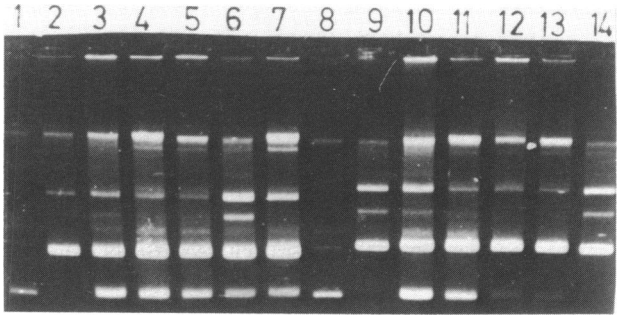


Figure 3. Displacement test. Gel electrophoresis of mini plasmid preparations from B.subtilis carrying plasmids pMER8 (lanes 1 and 8), pRB103 (lane 2) and pMET360 (lane 9) and from two different B.subtilis heteroplasmid strains. The strain carrying pMER8 and pRB103 was grown in double selective medium (lane 3) or in successive overnight cultures under non-selective conditions (lanes 4-7) as well as the strain carrying pMER8 and pMET360 (lane 10: double selective medium; lanes 11-14: successive overnight cultures under non-selective conditions).


4139
TTACTTCCAAAATTCAAATTTTCG:TTGCCAAAAATTAATCTGCTTTTG:CAAATTTTCTTCGTTATCCGTCAA
StopLysTrpPheGluPheLysArg GlnTrpPheAsnIleGlnLysGln LeuAsnGluGluAsnAspThrLeu
4211
AGTAGATTTCATTAAATCAGTTAA:ATCAATTGAAGATATTTCTTGTAT:TATTTGTTTATATTTGCGACGAGA
ThrSerLysMetLeuAspThrLeu AspIleSerSerIleGluGlnIle IleGlnLysTyrLysArgArgSer
4283
Dra I
ATTTCTATGTAATTCTCCCCATTT:GCTTTCTTCATGAAGTAATAAATA:AACCATTGCTTGTTCTTTTAAACT
AsnArgHisLeuGluGlyTrpLys SerGluGluHisLeuLeuLeuTyr ValMetAlaGlnGluLysLeuSer
4355
TTCCAAAGTAGCCCATGCAGGTTT:CAAAATGTGTAAGTCATTAAAACA:ATTATTCCAATAATCTACCATATC
GluLeuThrAlaTrpAlaProLys LeuIleHisLeuAspAsnPheCys AsnAsnTrpTyrAspValMetAsp
4427
TCGTTTAAGTTCAATTTCAACACG:CCATAGATGTTCAGCACTTACATC:AACATCTGCATTTTCTTTACGTTC
ArgLysLeuGluIleGluValArg TrpLeuHisGluAlaSerValAsp ValAspAlaAsnGluLysArgGlu
4499
TTTTTTCTTATTATAAATTCTAAT:AAATCTATTACTATCACGTGAGCC:AAAATATTTTGTTTCTGCCTTACC
LysLysLysAsnTyrIleArgIle PheArgAspSerAspArgSerGly PheTyrLysThrGluProLysGly
4571
Dra I,Hind III,Alu I
AGTTGTTCCGAAAAATACAGTTCG:CTTTAAAGCTTTTTCTGACAATGC:ATAATAATCGCTTAAATCATCTTC
ThrThrGlyPhePheValThrArg LysLeuAlaLysGluSerLeuAla TyrTyrAspSerLeuAspAspGlu
43
Alu I
AAAATCAAAAGCTAAATCTAATCT:TGTAAAACCATCATCTTCCATATA:ATCAATGATATTATGTTTTAACCA
PheAspPheAlaLeuAspLeuArg ThrPheGlyAspAspGluMetTyr AspIleIleAsnHisLysLeuTrp
115
TAACATCTCATCATGTGTAAGTTT:GTTGGGATTAAACTCAACTCTCAT:GTTACGTCTATCCCAAGTATCTGC
LeuMetGluAspHisThrLeuLys AsnProAsnPheGluValArgMet AsnArgArgAspTrpThrAspAla
187
Alu I,Dra I
TTTTACTTTGTCATATTCAATATA:AACTTTTTCTTGTAGTGCTTTAGC:TTTAAATTTTGTTTGAAGTATATC
LysValLysAspTyrGluIleTyr ValLysGluGlnLeuAlaLysAla LysPheLysThrGlnLeuIleAsp
259
Mbo I
Hae II
CCAAAGTCTAATTTGTGGATCTAA:ACTCATAAAATCGGATAGCTTTTT:AGCGCTGTTTTTATTAAGGTTTCC
TrpLeuArgIleGlnProAspLeu SerMetPheLysSerLeuLysLys AlaSerAsnLysAsnLeuAsnGly
331
Alu I
Hpa II
GACTATCGTCATAGCGTCAAAGCT:CAATTTCGGATTAGAAGTGCACAC:GAAATGTGCGTCTAACCGGCTATT
ValIleThrMetAlaAspPheSer LeuLysProAsnSerThrCysVal PheHisAlaAspLeuArgSerAsn
ori
403
Hpa II
Hinf I
Dde I
AGAGTAGCCGGTTTTAGAAAAATT:GTCTGAATCGTGATTTTCTAAGTG:ATTTGAATGATTTGTATAATTATT
SerTyrGlyThrLysSerPheAsn AspSerAspHisAsnGluLeuHis AsnSerHisAsnThrTyrAsnAsn
ori
Rep M
475
Taq I,Hinf I
Hph I
- 35
TTTACTCATAAAAAATCGACTCCT:TAATTTAATTTAAGAAGTCGCTCA:CCCGAATATATATCTTGAAGAATA
LysSerMet
S.D.
anti-S.D.
547
- 10
Xba I
cop RNAs
AACTAATATCGTTTAATATCTAGA:TATACAAATTAAGAACAAAAACAT:CAACTGTTTTTCTTTAAGGTAAGT
TATGTTTAATTCTTGTTTTTGTA GTTGACAAAAAGAAATTCCATTCA

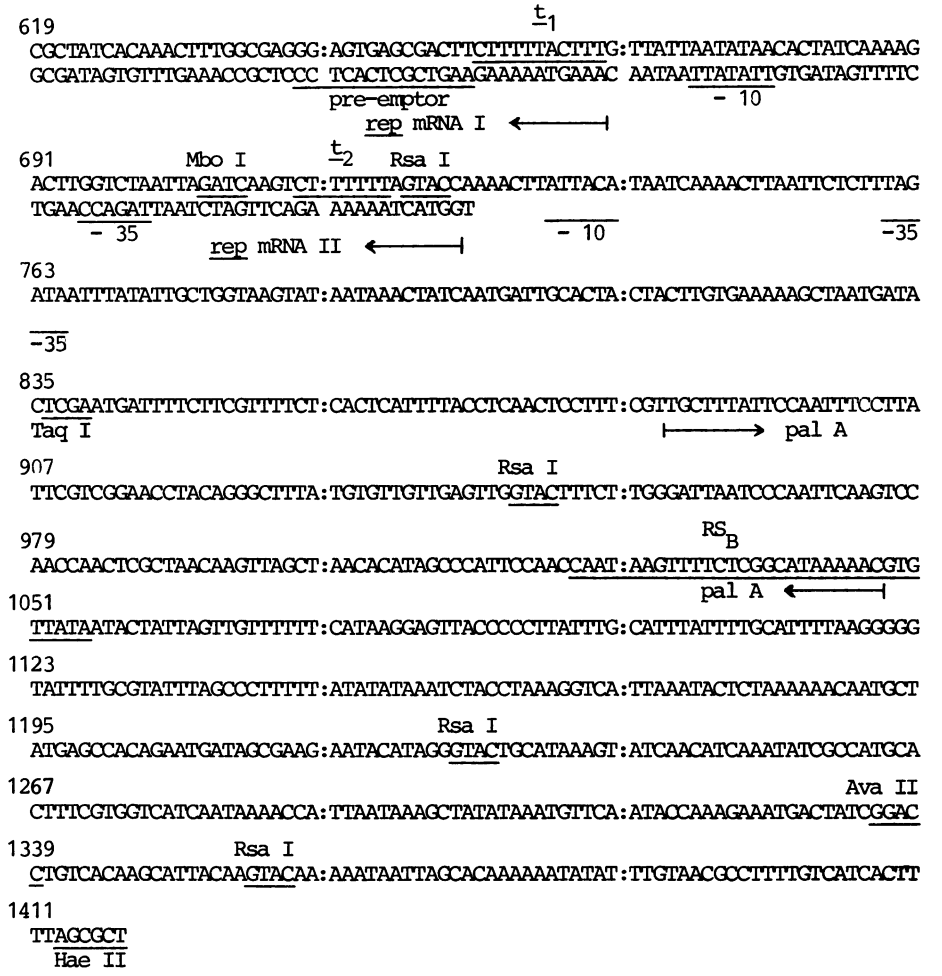


Figure 4. DNA sequence of pC223, pos. 4139-1418, HindIII taken as point of reference and plasmid size supposed to be 4.6 kb. The upper strand is written in 5' to 3' direction, and only the sequence coding for the cop RNAs is given double-stranded. For explanation of the indicated functions "anti-S.D.", "pre-emptor" and "RS_B" see discussion.

locate promoters on the fragment it was cut by the restriction enzymes RsaI, XbaI or MboI and used as template for run-off transcription. As can be seen in Fig.5, strong signals were obtained using the RsaI- and MboI-fragments for the copRNA run-off transcripts, and only weak signals with the XbaI-cut DNA for the rep mRNA run-off transcripts. Based on the results, a transcriptional map can be drawn for the TaqI fragment analogous to pT181 (4) (Fig.6) .

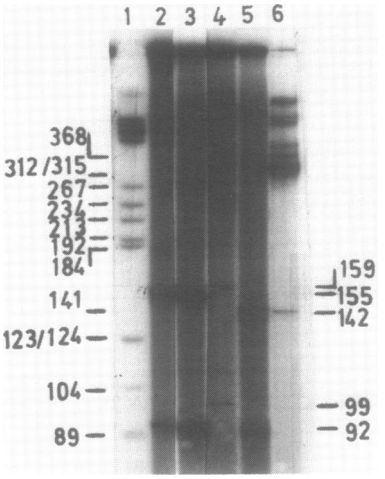


Figure 5. In vitro transcription of the 346 bp pC223-TaqI fragment, uncleaved (lane 2) and restricted by RsaI (lane 3), XbaI (lane 4) or MboI (lane 5). Aliquots of 3 µl were analysed by electrophoresis on an 8% polyacrylamide/8M urea gel, the bands being visualized by autoradiography. Transcript sizes (indicated in nt at the right) were determined by comparison with radioactive HaeIII- (lane 1) and TaqI- (lane 6) fragments of pBR322 (indicated in nt at the left).

Restriction maps of the basic replicons of pUB112, that has been physically and functionally analysed before (14), and pC223 show great homologies. Accordingly, in vitro transcription of pUB112, of its deletion derivative pRB311 (see above) and of a 260 bp pUB112-TaqI fragment, which is homologous to the 346 bp pC223-TaqI fragment, yielded also two small RNAs, 150 and 87 nt long (34). The nucleotide sequence of the corresponding DNA was determined as well as that of the ori region of pUB112 (see discussion).

Furthermore, dinucleotide-primed in vitro tanscription was performed using pC223 and pUB112 plasmid DNA as template to localize the copRNA initiation

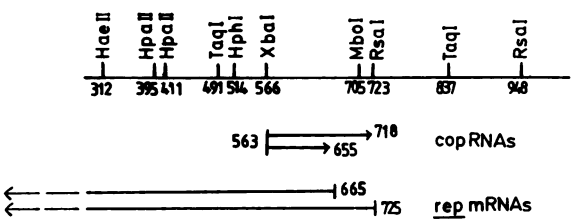


Figure 6. Transcriptional map of the 346 bp pC223-TaqI fragment.

Table 1. Copy numbers and segregation rates of several S.aureus plasmids in B.subtilis.

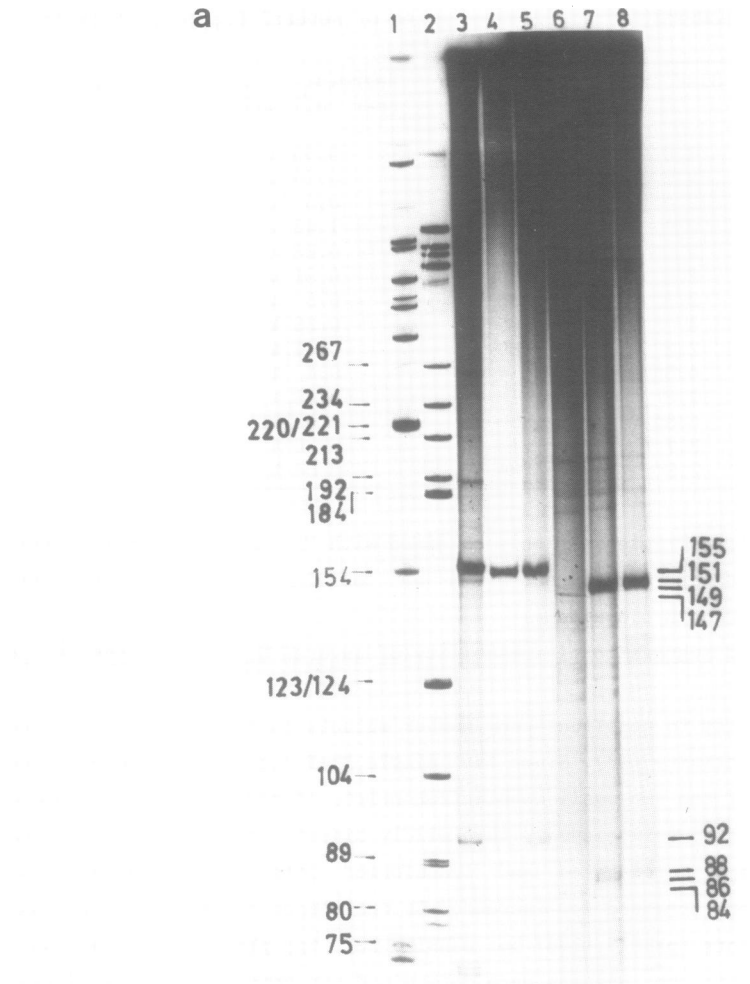
Plasmid	Size in kb	Copy Number (per cell)	Segregation Rate (per cell and generation)
pC223	4.6	9	0.69 %
pMEH25	3.2	55	0.26 %
pMET9	2.4	80	0.5 %
pXBE10	2.4	230	1.43 %
pXBE11	2.4	160	0.88 %
pXBE18	2.4	300	0.91 %
pMER8	2.15	25	0.3 %
pUB112	4	65	0.36 %
pRB311	2.2	80	0.34 %
pC221	4.55	4	0.4 %
pCW41	1.8	18	0.88 %
pUB110	4.55	50	0.00 %
pE194	3.7	13	0.34 %
pC194	2.9	35	0.04 %

sites. Counter-transcripts were only obtained with UpA and ApU, and the first nucleotide could be determined as A by priming with increased ATP concentrations for both plasmids.

Copy numbers and segregational instability of several S.aureus plasmids in B.subtilis

One possibility to construct copy number mutants is the alteration of the copRNA promoter activity (36). Since the single XbaI-site is located near the promoter region of the two small countertranscripts of pC223 (Fig.4), plasmid pMET9 was cleaved by this enzyme, then mildly digested by Bal31, connected with EcoRI-linkers, religated and transformed into B.subtilis BD 170. Transformants were screened for increased Cm resistance by replica plating. Thus, three mutants (pXBE10, 2367 bp; pXBE11, 2371 bp; pXBE18, 2367 bp) could be isolated. Their copy numbers were determined and compared with wild-type plasmid pMET9 (Table 1). In vitro transcription studies yielded shortened copRNAs of 147, 149 and 151 nt for pXBE10, pXBE11 and pXBE18, resp. (Fig.7a). Finally, the mutants were analysed by DNA sequencing of the regions affected by the mutations (Fig.7b). The results show that deletion of a few bp and insertion of the EcoRI-linker in the promoter- and the 5'-region of the two small RNAs has a profound effect on copy number, proving their involvement in copy number control, and indicate that the starting nucleotide of pC223 copRNAs is the A between two T's at position 572, not the one at 564.

Table 1 presents the copy numbers and segregation rates of pC223, of its deletion derivatives and of pMET9 copy number mutants in comparison with pUB112, pRB311 (see above) and some more S.aureus plasmids in B.subtilis.


a
1 2 3 4 5 6 7 8
267
234
220/221
213
192
184
154
123/124
104
89
80
75
155
151
149
147
92
88
86
84

b


Xba I
pMET9 533 TATCTTGAAGAATAAACTAATATCGTTTAATATCTAGATATACAAATTAAGAACAAAAACA 593
- 35
- 10
cop RNAs
Eco RI
pXBE10 533 TATCTTGAAGAATAAACTAATATCGTTTAATATCTAG CGGAATTCCG CAAAAACA 587
pXBE11 533 TATCTTGAAGAATAAACTAATATCGTTTAATAT CGGAATTCCG ATTAAGAACAAAAACA 591
pXBE18 533 TATCTTGAAGAATAAACTAATATCGTTTAATAT CGGAATTCCG AGAACAAAAACA 587

Interestingly, only pUB110 is stably maintained in the foreign host, whereas all the other plasmids exhibit segregational instability to varying degree, which does not appear to be correlated with copy number or plasmid size. Surprisingly, the copy numbers of the pT181-family members and their derivatives show large differences despite of their nearly identical basic replicons.

DISCUSSION

The comparison of pC223 and pUB112 at the nucleotide sequence and functional levels with two other well studied antibiotic resistance plasmids from S.aureus, pT181 and pC221, reveals their strong homology: The replication origin is located in the amino terminal region of the coding sequence for the replication initiation protein, and the copy number control system, similar to that of the gramnegative Inc FII plasmid family (7), is encoded in the region just 5' to the rep gene and utilizes two inhibitory countertranscripts.

The ori sequences of the pT181-group members all show a potential secondary structure, able to form three palindromic segments each containing a loop (1,7). For pT181 this palindromic region represents the binding site for the replication protein, a site-specific topoisomerase, the binding being centered on palindrome III (37). The heterology of the four palindrome III-loop sequences (Fig.8) supports the assumption that there the recognition specificity of the ori for the plasmid-specific Rep protein is located (7), and the great homology of the palindrome II-loop sequences (Fig.8), where the RepC nicking site of pT181 is located (8), suggests that the nicking site of Rep proteins is sequence - but not plasmid - specific. The pC223 ori was mapped by cloning in an E.coli vector and shown to be a function of segregational incompatibility in B.subtilis like inc3B of pT181 in S.aureus (32), what can be explained by competition for Rep molecules between plasmids carrying the same ori. Comparison of the nucleotide and amino acid sequences

Figure 7. (a) In vitro transcription of supercoiled plasmid DNAs of pC223 (lane 3), pMET9 (lane 5), pXBE10 (lane 6), pXBE11 (lane 7) and pXBE18 (lane 8) and of the 346 bp pC223-TaqI fragment (lane 4). As markers radioactive HinfI- (lane 1) and HaeIII- (lane 2) fragments of pBR322 were used. Transcript and marker fragment sizes are indicated as in figure 5.
(b) DNA sequences of the copRNA promoter regions of pMET9 and its copy number mutants. The indicated potential copRNA transcription initiation sites of the copy number mutants were estimated according to transcript sizes. As linker, a 10-mer Eco RI linker CGGAATTCCG was used. Only the upper strand is shown in 5' to 3' polarity.

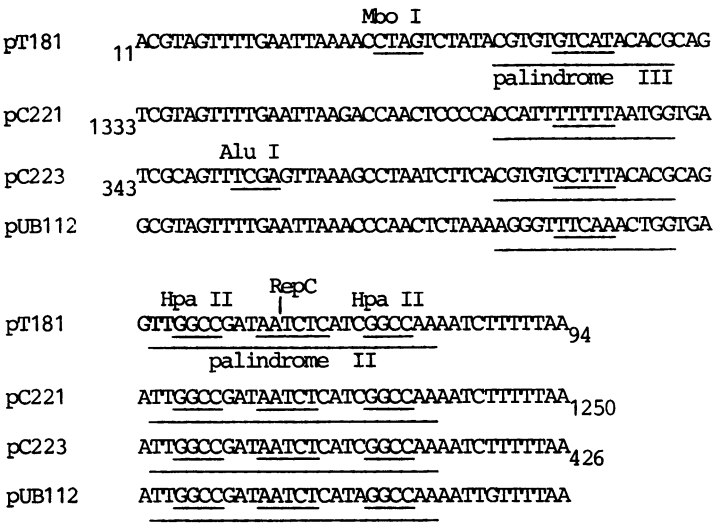


Figure 8. DNA sequences of the ori regions of plasmids pT181, pC221 (according to Projan et al., 7), pC223 and pUB112. Only the lower strand is drawn in 3' to 5' direction. The loops that are localized within the palindromic regions II and III are underlined. The nicking site of pT181 RepC (8) is also indicated. No exact positions for pUB112 can be given because this plasmid has not yet been totally sequenced. Based on the restriction map (14) and assuming that the second HpaII cut is located at position 1320 the presented sequence reaches from the approximate positions 1390 to 1300.

of pC223 RepM with those of the replication proteins from pT181 and pC221 exhibited an overall homology of approximately 80 percent. A region of great heterology is located towards the 3'terminus of the rep gene (between position 4349 and 4366 for pC223), what supports the notion put forward by Projan et al. (7) that here the ori recognition specificity of the plasmid-specific Rep proteins is encoded.

Another region of considerable nucleotide sequence homology is located in the rep leader sequence; it contains critical features of the model of negative regulation of rep mRNA translation by interaction with copRNAs, as proposed by Projan et al. (7). In Fig.9 a comparison of these features between four plasmids of the pT181-family is presented. It is apparent that the sequences called "SD", "anti-SD", "pre-emptor", the copRNA promoter and terminators and one of the two rep promoters are highly conserved. Sequence divergence mainly resides in the 5'terminal part of the copRNAs pointing out the importance of this region for compatibility i.e. inhibitor target specificity of the different plasmids.

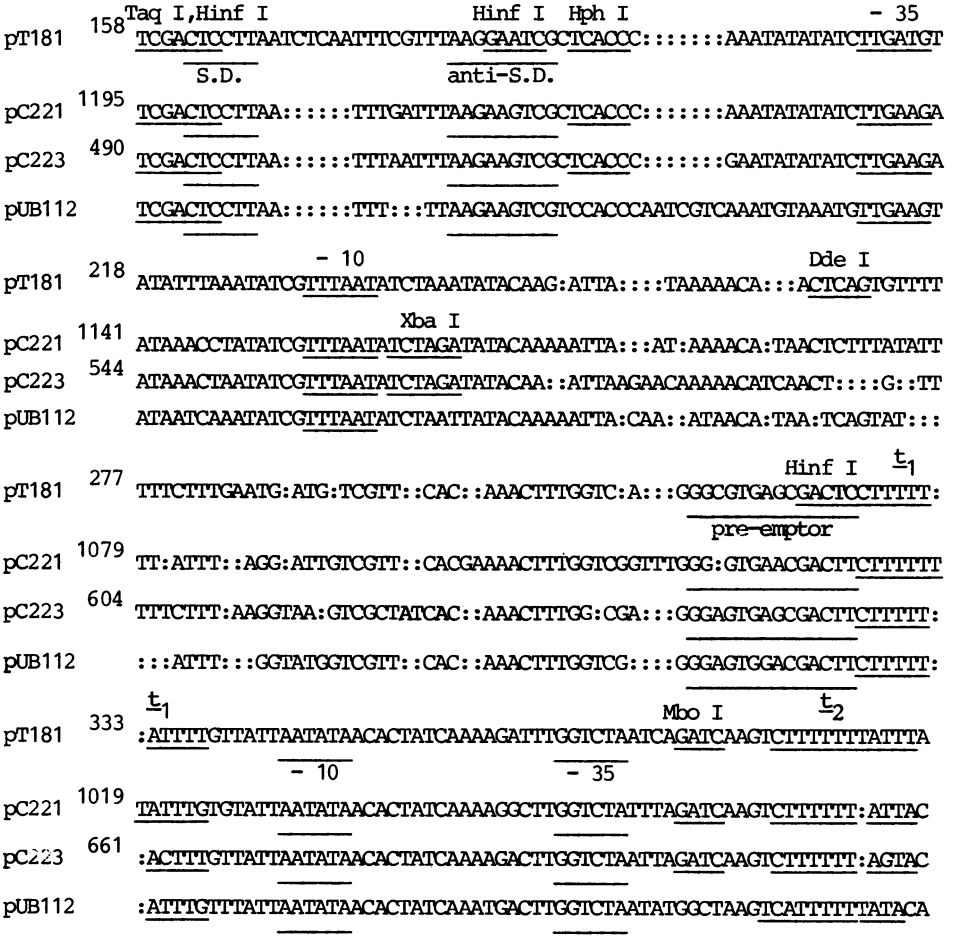


Figure 9. DNA sequences of the rep leader regions of plasmids pT181, pC221 (according to Projan et al., 7), pC223 and pUB112. Only the upper strand is shown in 5' to 3' polarity. The sequence of pUB 112 reaches from the approximate positions 1240 to 1000 (cf. legend to Fig. 8).

The role of copRNAs as a function of unilateral incompatibility by inhibiting plasmid replication in trans was shown for pT181 (inc3A) in S.aureus (23) and by us for pC223 in B.subtilis by displacement testing using the 346 bp TaqI fragment (see above). By deletion and exchange of nucleotides in this region the copy number of pMET9 in B.subtilis could be increased. In vitro transcription of the copy number mutants pXBE11 and pXBE18 yielded as much copRNAs as of pMET9, demonstrating that alteration of the 5'terminal copRNA region and not weakened copRNA promoter activity is responsible for

their increased copy number in contrast to pXBE10, *in vitro* transcription of which yielded less copRNAs (Fig.7a).

Just upstream of the *rep* gene but outside of the basic replicon of pC223, a 150 bp sequence, capable of forming a large imperfect palindrome, was detected analogous to pT181, pC221 and pS194, where it is called palA (35). The DNA sequences at both bases of palA are highly conserved, one arm being the common recombination site RS_B(38), and the total palA sequences of pC223 and pT181 at one and pC221 and pS194 at the other side are nearly identical, being classified as types I and II (1). PalA is required in *S.aureus* for normal rolling circle replication of pT181, pC221 and pS194 because of its function as lagging strand initiation site (35). Its rearrangement or deletion causes reduced plasmid copy number and stability in *S.aureus* (35). The palA-missing plasmids pMET9 and pMER8 replicate at higher copy numbers and are more stable in *B.subtilis* than their parent plasmid pC223 (Table 1). This lack of palA requirement in *B.subtilis*, also shown by Gruss et al. (35) for other pT181-family members, suggests the existence of another lagging strand initiation mechanism in the foreign host.

The comparison of six natural *S.aureus* plasmids exhibited great copy number differences in *B.subtilis* (Table 1), which can be explained for pUB110, pE194, pC194 and the pT181-group plasmids by different basic replicons and copy number regulation mechanisms (4,9,39,40). The differences within the pT181-plasmid family are likely to be due to functions outside of the homologous basic replicons, because deletions of those functions have a strong copy number-increasing effect for pC221 and pC223 but not for pUB112 (Table 1). The relatively low copy number of pMER8 is presumably caused by the deletion of one of two *rep* promoters, and the slightly higher copy number of pRB311 than that of pUB112 can be explained by the decreased amount of plasmid DNA. The functions outside of the basic replicon of pC221 that influence its copy number in *B.subtilis* negatively might be two open reading frames, shown to be necessary for the relaxation complex of this plasmid in *S.aureus* (7). Since pC223, in contrast to pUB112, exists also as a relaxation complex in *S.aureus* (12), the same is supposed for this plasmid.

The comparison of segregation rates exhibited pUB110 as the only stably inherited plasmid in *B.subtilis*, whereas all the others are unstable, the extent not being correlated to copy numbers. The stability of pUB110 in *B.subtilis* may be caused by a very efficient lagging strand synthesis (1), whereas unperfect lagging strand replication of the other plasmids in *B.subtilis*, probably caused by nonfunctioning of palA, seems to be the reason

for their instability. This view is emphasized by the observation that pUB110, in contrast to the other plasmids, does not produce single stranded circular plasmid DNA (41) as an intermediate of rolling circle replication (42), neither in S.aureus nor in B.subtilis.

The very weak segregational instability of pC194 may be due to its diffusible seg gene product, that is responsible for maintenance of this plasmid in B.subtilis (43). The decreased stability of pMET9 copy number mutants indicates that rearrangement of the copy control system raises plasmid instability in B.subtilis. We can conclude, therefore, that the instability of S.aureus plasmids in B.subtilis depends on the particular plasmid replication, partitioning and copy number control mechanisms.

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