Mutagenesis of the rpoS gene involved in alteration of outer membrane composition

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ABSTRACT

Background and Objectives: rpoS is a bacterial sigma factor of RNA polymerase which is involved in the expression of the genes which control regulons and play a critical role in survival against stresses. Few suitable vectors are available which could be maintained successfully in Flexibacter chinesis cells and could in particular be used as a suicide vector to make mutation in the rpoS gene. The aim of this study was to investigate if rpoS mutagenesis has impact on bacterial morphology in addition to cell division.

Materials and Methods: A 0.603 kb BamHI-PstI fragment subclone of pCRP0S38Ω was cloned into linearized pLYLO3. The final construct, pLRPOS38 suicide vector, was introduced into Flexibacter chinesis. Then the cytoplasm of mutant strain and wild-type were investigated by transmission electron microscopy.

Results: After successful subcloning of suicide vector into F. chinesis, based on TEM study, it was demonstrated that mutation in rpoS gene leads to decomposition of outer membrane of F. chinesis.

Conclusion: A suitable vector to make suicide mutation in rpoS was constructed for F. chinesis.

Keywords: rpoS; Flexibacter chinesis; Transposon; Suicide vector; Transmission electron microscopy

INTRODUCTION

rpoS controls a large regulon (1, 2) and plays a crit-
The rpoS gene is located in a structural gene with one operon common with nlpD encoding a lipoprotein. There is two promoters not regulated by growth phase and in experiential growth phase demonstrated a low level of rpoS expression (5). The rpoS transcription is controlled by the cAMP-CRP complex negatively. There is a transcriptional start site for rpoS gene, however there is no report of involvement of this site in rpoS transcriptional control (15, 16). Every attempt to manipulate a suitable construct, using the conventional cassettes and suicide vectors, which usually are used with E. coli and other Gram-negative bacteria failed.

In this study a new technique was reported which proved suitability for the genetic manipulation of gilding bacteria (17). In this study, the 5.97 kb pLYLO3 suicide vector was digested and a 0.603 kb Bam HI fragment subclone of pCRP038Ω was cloned into linearized pLYLO3. This construct was named pLRPO38 and introduced into Flexibacter chinensis using conjugation method. Then the cytoplasm of mutant strain and wild-type were investigated by transmission electron microscopy.

MATERIALS AND METHODS

Bacterial growth media and conditions. All bacterial strains were grown in Luria Agar (5 g/l NaCl, 15 g/l Agar, 10 g/l tryptone, 5 g/l yeast extract) or on Luria Broth (5 g/l NaCl, pH 7.2, 5 g/l yeast extract, 10 g/l Bacto tryptone).

Amplification of the rpoS gene. Primers which were as follow: 5ʹGGGGGATCCGTCAGAGGAT-CAGGGTAGGAGCCAC3ʹ (forward), and 5ʹGG-GGAATTCTTCAACCTGAATCTGGCAGAAC-GTTAC3ʹ (Reverse) were used to amplify the rpoS gene in PCR assay. Amplification was carried out using High Fidelity PCR Master Kit (Roche) and a Perkin-Elmer (USA) DNA thermal cycler. Amplification conditions were 94°C for 45 sec (1 cycle), 94°C for 1 min, 65°C for 45 sec, 72°C for 30 sec (30 cycles). The PCR products were purified using High Pure Product Purification Kit (Roche).

Conjugation method. The method described by Maniatis et al. 1989, was used with slight modification. E. coli S17-1 was used as the donor for the transfer of the recombinant plasmid, pLRPO38 into F. chinensis. The plasmid RP4 is an IncP type plasmid which is integrated into the chromosome of E. coli S17-1 and the plasmid pLYLO3, contains an oriT (transfer origin) from PK2, an incP1 plasmid, which is recognized by IncPα plasmids, such as RP4, but not by IncPβ plasmids. The recombinant plasmid, pLRPO38, was transferred into the E. coli S17-1 by transformation and the recombinant E. coli S17-1 was used as donor to introduce the recombinant plasmid, pLRPO38 into F. chinensis as recipient, using bi-parental conjugation method. Then the trans-conjugated bacteria were cultivated onto LB agar containing 200 μg erythromycin to select for trans-conjugants. The plates were incubated for 2 to 3 days at 30°C (18, 19).

Transmission electron microscopy. Samples were prepared and examined using the Jeol JEM-100S transmission electron microscope with an 80 kV accelerating voltage. Photographs were taken using Kodak Panasonic film, which was developed in Kodak D-19 developer at 20°C for 3 min and fixed in Kodak fixer.

RESULTS

Strategy for mutagenesis in the rpoS gene of F. chinensis. The strategy for the mutation of the rpoS gene was the interruption of the gene by the insertion of an interposon (Ω) into the gene. A one kb EcoRI/BamHI fragment of the rpoS gene subclone of pBRPO38 was cloned into pUC19H which provided some convenient poly cloning site for the construction. The construct was named pICRPO38. The restriction map of the miniprep of the rpoS gene in this construct is shown in Fig. 1a. A unique Pml I site was approximately in the middle of the gene and unique for the cloning of pICRPO38. The restriction digestion of Pml I site approximately located in the middle of the rpoS gene is shown in Fig. 1b. This site was used for insertion of a 0.8 kbp interposon into the rpoS gene.

Interruption of the rpoS gene of F. chinensis. A 0.8 kb Ω fragment consisting of the CM (chloramphenicol) resistance gene of pKR810 plasmid was digested with Hinc II (which has recognition sites at both ends of the cassette) and purified. The constructed pICRPO38 was linearized by diges-
tion at the unique Pml I site. Both the linearized pICRPOS38 and Ω fragments were blunt ended through the digestion with Hinc II and Pml I. The Ω fragment was ligated into the Pml I site of pICRPOS38 yielding pKRPOS38Ω. This was transformed into E. coli TG1 and selection for CM resistance was made which confirmed that the Ω fragment had been inserted into the rpoS gene. pKRPOS38Ω was digested with BamHI with four sites. A nondigested sample was used as the control. In Fig. 2 the 0.8 kb bands in lanes 2 and 4 belong to the Ω fragment.

Subcloning of the rpoS mutation into the suicide vector, pKNG101. The mutated rpoS gene with the interposon digested from pICRPOS38Ω at the BglII and BamHI sites was introduced to a linearized pKNG101 digested at a BamHI and dephosphorylated to prevent self-ligation. The linearized vector and rpoS mutant gene were ligated and the construct was named pKRPOS38Ω. Restriction analysis was carried out to confirm the presence of a 1.8 kb fragment of the mutated rpoS gene in pKRPOS38Ω. As the BglII and BamHI sites had the same ends, they would ligate but neither BamHI nor BglII recognized the BamHI and BglII ligated site. pKRPOS38Ω was digested using four enzymes in two separate digestions. At first, digestion was carried out with Sall and BamHI. Due to the orientation of the insertion in the vector, two possibilities could occur with this digestion. Either the BglII downstream of the mutated rpoS gene could be ligated into the BamHI site located next to the Sall site in the vector, subsequent digestion of Sall and BamHI miniprep would result in a 1.8 kb insertion and 6.8 kb vector band; or the BglII end could be ligated to the BamHI site downstream of the rpoS mutated gene located next to BstBI of the vector, then digestion at the Sall and BamHI sites would not result in any bands. The digestion of Sall and BamHI resulted in 1.8 kb fragment which belonged to the mutated rpoS gene and 6.8 kb vector fragment which showed that the first possibility had occurred here (Fig. 3).

Introduction of plasmid pKRPOS38Ω into F. chinesis cells. Initially electroporation was attempted to transfer the DNA into F. chinesis. Subsequently, conjugation was employed for the introduction of plasmid by tri-parental mating. Every attempt to introduce pKRPOS38Ω by electroporation was unsuccessful, probably because of the restriction

Fig. 1. a) Miniprep of pICRPOS38 was digested with a number of restriction enzymes. Lane 1 is λ ladder of 1 kb DNA as a molecular size. Lane 2 is pICRPOS38 digested with EcoRI and BamHI b) Restriction digestion of pICRPOS38 at a unique PmlI site. Lane 1 is λ ladder of 1 kb DNA as a molecular size. Lane 2 is pICRPOS38 digested with PmlI. Lane 3 is pICRPOS38 digested with PmlI and EcoRI. Lane 4 is pICRPOS38 digested with PmlI and BamHI. Lane 5 is pICRPOS38 digested with PmlI, EcoRI and BamHI.

Fig. 2. Restriction digestion of pICRPOS38Ω. Lane 1 is λ ladder of 1 kb DNA as a molecular size. Lane 2 is pICRPOS38Ω digested with PstI. Lane 3 is undigested pICRPOS38Ω as a control. Lane 4 is pICRPOS38Ω digested with HindII and Lane 5 is pICRPOS38Ω digested with BamHI.
of transferred DNA by the *F. chinesis* restriction system. Conjugation was widely used to transfer pKRPOS38Ω into *F. chinesis* cells by a tri-parental mating method. One of the three parents was *E. coli* GJ342 which contains the helper plasmid, JC2926; the second parent was *E. coli* SM10::pir. The construct can only replicate in this strain because it supplies in Trans form of the π protein encoded by the *pir* gene, such as in the *E. coli* strain lysogenized with a *pir* transducing λ phage. All the attempts using conjugation to transfer pKRPOS38Ω into the *F. chinesis* were unsuccessful due to unknown factors.

Introduction of some plasmids, such as pBlue+, into *F. chinesis* was attempted alongside *E. coli* ML30 as a control. Electroporation, different methods of transformation and conjugation were used to transfer this plasmid into *F. chinesis*. Although all the methods were highly successful with the control, no *F. chinesis* colonies were found. The positive control proved that the transformation methods were successful, but due to unknown factors the plasmid was not maintained in *F. chinesis*.

**Introduction of Tn4351 into *F. chinesis*** on the broad-host range IncPβ plasmid R751 (R751::Tn4351). Attempts were made to introduce Tn4351 on the broad-host ranged Incβ plasmid R751 (R751::Tn4351) into *F. chinesis*. A construct of R751::Tn4351 was selected for introduction into *F. chinesis* to discover if the introduction and insertion of the vector R751 and the transposition of T4351 into the *F. chinesis* chromosome by a tri parental mating occurred. One parent was *E. coli* GJ342 which carried a helper plasmid, the second parent was *E. coli* HB101 which contained R751::Tn4351 and the third parent was the *F. chinesis* target strain. 189 colonies were isolated on LB agar plates which in passage in fresh media were able to grow in 200 µg/ml erythromycin. The erythromycin resistance gene is carried by Tn4351. Erythromycin resistance colonies were transfer to LB agar containing 200 µg/ml trimethoprim. None of the colonies could grow in this medium and no free vector (R751) was obtained in plasmid miniprep. This indicates that no replication of R751 occurred. Colony blot hybridization was done separately to discover if Tn4351 and/or R751 had inserted into the chromosome of *F. chinesis*. Duplicated blots were probed separately with radio-labeled pVOHI (for the detection of the Tn4351) and R751 (for the detection of the transposon delivery vector). pVOHI is a derived of pBR328 that carries Tn4351 but there is no common sequence with R751. Approximately half of the colonies selected from the first screen were positive in the second screen for the detection of Tn4351 (Fig. 4.a). A few colonies were positive for the detection of R751 (Fig. 4.b). A few mutant defective in spreading were isolated (Fig. 4.c) and some auxotroph mutants were also isolated.

**Insertional mutagenesis in *F. chinesis*** using the novel pUC19-based suicide vector (pLYO3). After successfully cloning Tn4351 into *F. chinesis* and isolation of some auxotroph and none spreading mutants, a convenient suicide vector was chosen for disruption mutagenesis in *F. chinesis*. The identification of the *ermF* gene which was carried by Tn4351 and conferred erythromycin resistance in *F. chinesis* was an efficient method for showing that conjugation from *E. coli* to *F. chinesis* had been taken place, pLYO3 and pUC19 based suicide vector for *Bacteroides* spp., contained *ermF* and also an origin of transfer which would allow conjugative transfer from *E. coli*. The *ermF* gene was not expressed in *E. coli* (20). pLY03 was constructed originally from pFD160, which contains pUC19 and the cryptic bacteroides plasmid pBI143 and was the basis for the
GUC vector. pBI143 also contained a mobilization region that was recognized by the IncP plasmid R751. The region encoding the GUS on a 1.9 kb fragment was removed from pBT101 and cloned into pFD160 which had been digested with BamHI and SstI and named pMJF-1. pMJF-2 was constructed by inserting a 2.9 kb ClaI fragment containing the ermF gene from the bacteroides transposon Tn4351 into pMJF-1 (the 2.9 kb fragment was digested from a shuttle vector pVIC-1). The pBI143 segment from pMJF-2 was deleted and replaced with an oriT region from RK2. oriT was recognized by IncPα plasmid, such as RP4, but not by IncPβ plasmids, such as R751. This construct was named pCQW1 (21). Finally a 1.9 kb SmaI-SstI of the region encoding GUS was deleted from pCQW1 and the pCQW1 lacking the SmaI-sstI site was named pLYO3 (22, 23). Therefore, the 5.9 kb pLYO3 suicide vector was digested with BamHI and PstI sites and a 0.60 kb BamHI-PstI fragment sub-clone of pCRP38Ω was cloned into the linearized pLYO3 digested with BamHI-PstI. Fig. 5 shows the results of the digestion of the 0.603 kb BamHI-PstI fragment from the construct, pLRPOS38.

**Introduction of the construct pLRpOS38 into F. chinesis cells by conjugation.** The construct,
pLRPOS38 which contained oriT to allow conjugation was transferred into *F. chinesis* by a bi-parental conjugation method that one of the parents was *E. coli* S17-1 which contained a derivative of RP4 (RP4-2::Mu-Km::Tn7Tc') integrated into the chromosome (24, 25). The other parent was *F. chinesis* as a recipient strain. The construct containing oriT carried the specific mob site which was recognized by IncPα plasmids, such as RP4, and facilitates transfer of the construct from *E. coli* S17-1. It also contained an ampicillin resistance gene which was expressed in *E. coli* and *F. chinesis* and an erythromycin resistance gene which was not expressed in *E. coli* but was expressed in *F. chinesis*. Therefore as the ermF gene did not confer erythromycin resistance to *E. coli* strain, the transformed construct in *E. coli* S17-1 was selected with ampicillin. After conjugation had taken place, as the *ermF* gene was not expressed in *E. coli*, the ex-conjugant cells were selected with erythromycin. 26 colonies were isolated on an erythromycin medium. This result demonstrated for the first time that pLYLO3 worked successfully as a suicide vector in gliding bacteria. The *rpoS*:pLYLO3 ex-conjugants were transferred into fresh erythromycin (200 µg/ml) medium to eliminate possible pseudo ex-conjugants.

**Selection of recombinant *F. chinesis*.** 24 erythromycin resistance colonies are single recombinants since the target gene was disrupted at one site. These cells would also been ampicillin resistant. The recombinant clone was *F. chinesis* carrying pLRPOS38 plasmid. Also the accuracy of cloning was confirmed by colony blot analysis (Fig. 4.a and 4.b).

**Study of cell morphology using transmission electron microscopy (TEM).** The recombinant *F. chinesis* cells had a quite different shape to the starved wild-type cells and no shrinkage in the cytoplasm was observed (Data not shown). TEM study showed that the mutant strain had no outer membrane. This result suggested that the *rpoS* gene was important for composition of outer membrane in *F. chinesis* (Fig. 4.c).

**DISCUSSION**

The genetic studies of gliding bacteria had been limited with the exception of studies on one species, *Myxococcus xanthus* (26). Mutant of *M. xanthus* which were defective in motility had been isolated and a number of genes identified which were necessary for colony spreading (17). Following the development of technique for the genetic manipulation of *Bacteroides* spp., a number of techniques were developed to genetically manipulate gliding bacteria (17, 27).

Tn4351 was originally isolated from *Bacteroides fragilis* (28). The transposon was successfully introduced into *Cytophaga succinicans*, *Flavobacterium meningosepticum*, *Flexibacter canadensis*, *Flexibacter* strain SFI and *Sporocytaphaga myxococoides* by conjugation (17). Tn4351 carried two antibiotic resistance genes. One of the genes coded for resistance to erythromycin and clindamycin was expressed in *Bacteroides* but not in *E. coli*. The other gene coded resistance in tetracycline and was expressed in aerobically grown *E. coli*, but not in anaerobically grown *E. coli* or in *Bacteroides*. The transposon of Tn4351 was originally detected in *E. coli* which carried an unstable chimeric plasmid, pSS-2.
The mobilization of pSS-2 from one strain of *E. coli* to another led to insertion of Tn4351 into R751 (R751: Tn4351) and less frequently to the insertion of Tn4351 into the chromosome. R751 also mobilized pE5-2 which contains a 3.8 kb EcoRI fragment of Tn4351 and can mobilize a cryptic bacteroides plasmid into *Bacteroides* spp. R751 was never detected in any of the *Bacteroides* trans-conjugants that carried pE5-2. This result demonstrates that R751 was not maintained in *Bacteroides*. In addition, there is some evidence that when R751 is inserted in to the bacteroides chromosome, it cannot conjugate out of the *Bacteroides*. Consequently it could be used as a suitable suicide vector in *Bacteroides* species.

Few suitable vectors are available which could be maintained successfully in *F. chinesis* cells and could, in particular, be used as a suicide vector to make mutation in the *rpoS* gene. In this study we used a novel method for mutagenesis of the *rpoS* gene in *F. chinesis*. Initially pKNG101 and pKRPOS38 plasmids were used to mutate the *rpoS* gene in the *F. chinesis* but the attempts were unsuccessful. Then in the bacteroides transposon, Tn4351 was cloned into R751 and transformed into *F. chinesis* successfully. In this method the 5.97 kb pLYLO3 suicide vector was digested and a 0.603 kb BamHI fragment subclone of pCRPOS38Ω was cloned into linearized pLYLO3. This construct was named pLRPOS38 and introduced into *F. chinesis* using conjugation method. The accuracy of cloning was approved by cultivating of recombinant strain onto media containing erythromycin and ampicillin and using southern blot analysis. Electron microscopy of starved wild-type and starved recombinant *F. chinesis* showed that the cytoplasm of starved wild-type cells became clear and shrunken leaving cells which had an odd shape and appearance. In recombinant *F. chinesis* the cells had a quite different shape to the starved wild-type cells and no shrinkage in the cytoplasm was observed, previously in *E. coli* it was reported by Ozkanka (1993) (29) and in *Hydrophila* by Lim (1995) (30).

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