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FEMS Microbiology Letters 188 (2000) 125–128

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

The *cyl* genes of *Streptococcus agalactiae* are involved in the production of pigment

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Received 16 May 2000; accepted 18 May 2000

Abstract

The *cyl* genes of *Streptococcus agalactiae* are required for the production of hemolysin. Based on the observation that nonhemolytic *S. agalactiae* mutants do not produce pigment, a close genetic linkage between hemolysin and pigment has been postulated. To investigate this genetic linkage and to identify genes involved in the production of the *S. agalactiae* pigment, we screened mutant libraries for nonpigmented clones. Four distinct mutants were isolated with a nonpigmented and nonhemolytic phenotype. The mutations had occurred either in known *cyl* genes or in two open reading frames located immediately downstream. These novel genes are cotranscribed with the *cyl* gene cluster and were designated *cylF* and *cylI*. Our data indicate that identical genes participate in the production of *S. agalactiae* hemolysin and pigment.

Keywords: Group B streptococcus; Hemolysin; Pigment

1. Introduction

Streptococcus agalactiae (group B streptococci, GBS) is a member of the genus Streptococcus with a yet poorly understood genetic background. Employing a novel mutagenesis strategy, our laboratory has recently identified a cluster of genes (*cyl*) that is essential for the production of the *S. agalactiae* hemolysin. Currently eight different *cyl* genes have been identified and described. The deduced proteins display similarities to ABC (ATP binding cassette) transporters and prokaryotic fatty acid biosynthesis enzymes [1]. *S. agalactiae* knock out mutants of the *cyl* genes *acpC*, *cylZ*, *cylA*, *cylB*, and *cylE* display uniformely both a nonhemolytic and nonpigmented phenotype. Several investigators have previously reported that nonhemolytic *S. agalactiae* mutants also lack pigment production [2,3]. The production of yellow-orange pigment by *S. agalactiae* is frequently used for diagnostic purposes [4,5] and has been investigated since the 1970's. Although a close genetic linkage between the production of the β-hemolysin and the pigment of *S. agalactiae* has been postulated for several years [6], the molecular structure of this pigment, or the genes responsible for pigment production, however, remain unknown. To investigate the genetic basis for pigment production and the linkage between hemolysin and pigment expression, pGh9:IS*S1* mutant libraries of two independent wild-type strains were screened for nonpigmented mutants.

2. Materials and methods

2.1. Bacterial strains

The *Escherichia coli* and *S. agalactiae* strains used in this study are listed in Table 1. *E. coli* strain XL1-Blue MRF (Stratagene, Heidelberg, Germany) was used as a host for the phage lambda ZAP express. *S. agalactiae* isolates were cultured on Columbia agar (Oxoid, Basingstoke, England) supplemented with 3% sheep blood, in Todd–Hewitt broth (THB) (Oxoid) or in THB supplemented with 0.5% yeast (THY) at 37°C. For the identification of nonpigmented *S. agalactiae* strains, pGh9:IS*S1* mutant libraries were cultured on Granada medium, which enhances production of the *S. agalactiae* pigment [7] Mutant strains harboring chromosomally integrated pGh9:IS*S1* vectors were subcultured in medium containing 5 mg l^{-1} erythromycin at a temperature of ≥ 37°C.

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0378-1097/00/$20.00
PII: S0378-1097(00)00224-X

2.2. DNA techniques

Standard recombinant DNA techniques were employed for nucleic acid preparation and analysis. Genomic streptococcal DNA was isolated as described previously [8]. Plasmid DNA was isolated and purified using the Qiaprep Spin Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out with Taq polymerase according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany) with 35 cycles of amplification steps of 1 min at 94°C, 1 min at 50–56°C and 1 to 3 min at 72°C depending on product size. Plasmids and PCR products were sequenced on an ABI 310 automated DNA sequencer using the ABI PRISM™ Big-Dye terminator cycle sequencing kit (PE Applied Biosystems, Weiterstadt, Germany). *S. agalactiae* strains were transformed according to the protocol of Ricci et al. [9]. Construction of the pGh9:IS*S1* mutant libraries in *S. agalactiae* strains O90R and AC475, identification of the insertion site, and excision of the integrated vector were performed as described by Maguin et al. [10]. A lambda ZAP express library of strain AC475 was used for the identification of nucleotide sequences located upstream and downstream of the initial pGh9:IS*S1* insertion sites. Screening of the lambda library was carried out as previously described [1].

2.3. RNA preparation and analysis

Total RNA from strain AC475 was prepared and reverse transcription (RT) was carried out as described previously [1]. For the RT a primer was used annealing at codon 146–138 of *cylI* (5′-CTC CCA CAA TAT CAT GAT AAG CC-3′). The subsequent PCRs with three different primer pairs (A: 5′-TAT TCT TGG AAT CTA GAT TTC AC-3′ and 5′-CTC CCA CAA TAT CAT GAT AAG CC-3′; B: 5′-AAG TTA CCC GAT TGA GCA TG-3′ and 5′-CTC CCA CAA TAT CAT GAT AAG CC-3′; C: 5′-TCT CTC CAA ATG GCA GCC-3′ and 5′-CTC CCA CAA TAT CAT GAT AAG CC-3′) were performed on RNA preparations prior to and following the RT reaction and on genomic DNA as control. The primer annealing sites for PCR reaction A are located on *cylF* and *cylI* and for reaction B and C on *cylE* and *cylI*, respectively.

2.4. Nucleotide sequence accession number

The nucleotide sequence of the coding regions for the *S. agalactiae cyl* genes has been submitted to the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries and was assigned the accession number AF093787.

3. Results and discussion

PGh9:IS*S1* mutant libraries of *S. agalactiae* wild-type strains O90R and AC475 were screened for nonpigmented mutant strains. Among several thousand colonies, four distinct nonpigmented mutants were isolated and analyzed (Table 1). The genetic characterization of the vector insertion sites revealed that in two of the four mutant strains mutations of previously identified *cyl* genes were found (Fig. 1). The first mutation was located in *cylD*, a gene with a deduced amino acid sequence similar to FabD of *Bacillus subtilis* [11]. The second strain harbored a mutation of *acpC* which codes for a protein that displays significant homologies to several acyl carrier proteins. In *cylD*, the pGhost plasmid was inserted 450 bp downstream of the start codon. Thus far, an *S. agalactiae* mutant strain

Table 1
Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strain		
E. coli		
Dh5α	*endA1 hsdR17 supE44 ΔlacU169 (φ80lacZDM15), recA1, gyrA96 thi-1 relA1*	Boehringer
XL1-Blue MRF	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI qZΔM15* Tn*10* (Tet^r)]	Stratagene
EC 101	*E. coli* JM101 derivative with *repA* from pWV01 integrated into the chromosome	Law et al. (1995) [16]
S. agalactiae		
AC475	clinical wild-type isolate, Hly$^+$	Aachen collection
PIG1	AC475 derivative *cylF::*pGh9:IS*S1*, Hly$^-$, Pig$^-$	This study
PIG1ex	AC475 derivative *cylF::*IS*S1*, Hly$^-$, Pig$^-$	This study
PIG5	AC475 derivative *cylD:*pGh9:IS*S1*, Hly$^-$, Pig$^-$	This study
PIG5ex	AC475 derivative *cylD::*IS*S1*, Hly$^-$, Pig$^-$	This study
PIG20	AC475 derivative *cyll::*pGh9:IS*S1*, Hly$^-$, Pig$^-$	This study
PIG20ex	AC475 derivative *cyll::*IS*S1*, Hly$^-$, Pig$^-$	This study
O90R (ATCC 12386)	R. Lancefield grouping strain, Hly$^+$	ATCC collection
PIGA	O90R derivative *acpC::*pGh9:IS*S1*, Hly$^-$, Pig$^-$	This study
PIGAex	O90R derivative *acpC::*IS*S1*, Hly$^-$, Pig$^-$	This study
Plasmid		
pGh9:IS*S1*	Ery^R, ori Ts	Maguin et al. (1996) [10]

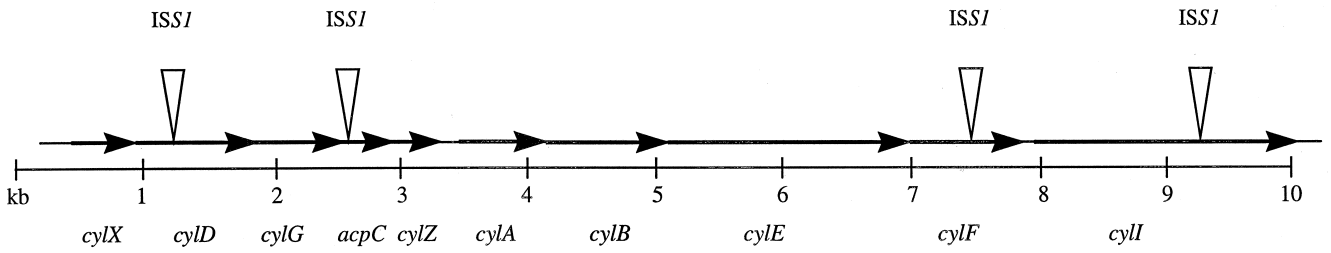

Fig. 1. Graphic map of the *cyl* gene cluster, pGh9:IS*S1* plasmid integration sites of the nonpigmented mutant strains that were isolated in this study are indicated as IS*S1*.

of CylD, a putative malonyl-CoA-ACP transacylase, has not been reported previously. Since mutants of this gene are viable, it is unlikely that *S. agalactiae* possesses only a single gene encoding an enzyme with a function critical for prokaryotic fatty acid biosynthesis. Our results support the hypothesis that CylD has a specialized function for the expression of hemolysin and pigment, and that the malonyl-CoA-ACP transacylase that is required for regular fatty acid biosynthesis is encoded by a different gene. Viable nonhemolytic and nonpigmented *S. agalactiae* mutant strains of *acpC* have been isolated previously which is consistent with the hypothesis that *acpC* encodes a specialized acyl carrier protein essential for hemolysin and pigment production [1].

The pGh9:IS*S1* insertion sites of the two remaining mutant strains occurred in open reading frames that are located downstream of the known *cyl* genes (Fig. 1). The deduced 36.7 kDa protein of the first of these reading frames was designated CylF. It consists of 317 amino acids and the putative protein has a 23% identity and 39% similarity to *gcvT* that encodes the aminomethyltransferase (glycin cleavage system T protein) of *E. coli* [12]. A typical ribosome binding site is located 11 bp upstream of the ATG start codon. The second downstream open reading frame designated *cylI* codes for a putative 79.4 kDa protein of 731 amino acids. Comparison with the GenBank database revealed a 32% identity and 54% similarity with the beta-ketoacyl-ACP-synthase II encoded by *fabF* of *E. coli* [13].

The location of *cylF* and *cylI* immediately downstream of the known *cyl* genes and the loss of pigment production and hemolysis in mutants of this locus suggested that these genes belong to the *cyl* gene cluster. To analyze if *cylF* and *cylI* are cotranscribed with *cylE*, transcription analysis was performed with RT-PCR. Following a reverse transcription that originated from *cylI* a PCR reaction was performed with primer pairs annealing on *cylF* and *cylI* (A) and on *cylE* and *cylI* (B, C) respectively. PCR products of 750 bp, 2.1 kb and 2.4 kb were generated using the RT reaction mixture as templates and from genomic DNA which served as a positive control. Total RNA that had not been subjected to an RT reaction was used as a negative control and did not yield any PCR products (Fig. 2). Our results suggest that *cylF* and *cylI* are located on a common transcript with *cylE*.

In previous studies on nonhemolytic *S. agalactiae* mutants, we identified four distinct IS*S1* mutants of *acpC* and *cylE*, two naturally occurring mutants of *cylA* and *cylB* and generated targeted mutants of *cylZ* [1,14]. In the current screen for nonpigmented *S. agalactiae* clones, novel mutant strains harboring insertions in the *cylD*, *cylF* and *cylI* genes were isolated. These findings led to the identification of two novel genes located downstream of the known *cyl* genes. All of the different mutant strains that could be identified in screens for nonhemolytic or nonpigmented mutants are both nonhemolytic and nonpigmented confirming earlier reports about a strong association between the hemolytic and pigmented phenotype of *S. agalactiae*. Moreover, we were not able to isolate a single mutant with an isolated deficiency of either hemolysin or pigment production. Based on the amino acid sequence similarities this cluster of genes appears to be responsible for the biosynthesis and export of a yet unidentified molecule that is essential for hemolysin and pigment production. Our data are also consistent with the hypothesis that

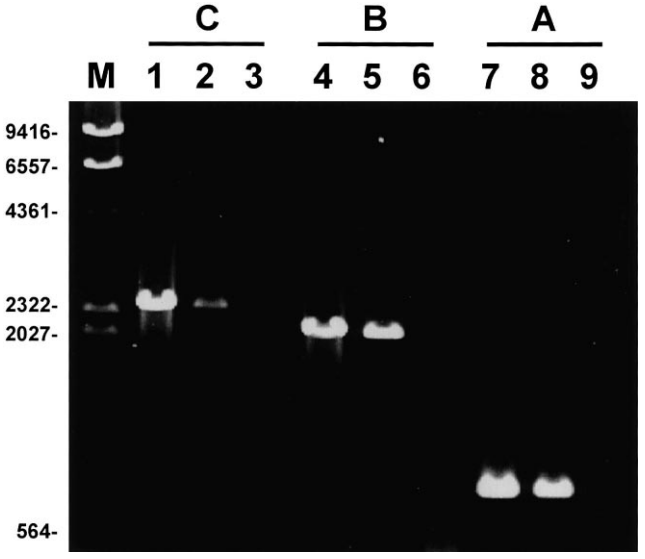

Fig. 2. Transcription analysis of *cylF* and *cylI* by RT-PCR. A primer annealing at codon 146–138 of *cylI* was used for reverse transcription. Subsequent PCR was carried out with genomic DNA as a positive control (lanes 1, 4, 7), mRNA subjected to an RT reaction (lanes 2, 5, 8) and mRNA without prior RT reaction (lanes 3, 6, 9) as a negative control. C, B, and A refer to the different primer pairs as mentioned in the text. Lane M, molecular mass marker (in nucleotides).

the hemolysin molecule is pigmented, as it is known for the hemolysin of *Legionella pneumophila* [15] or that the pigment is a derivative of the hemolysin. Taken together, our findings indicate that *cylF* and *cylI* belong to the *cyl* gene cluster and that the hemolysin and pigment share a common genetic background.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Sp 511/4-1) to B.S.

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