A divIVA null mutant of *Staphylococcus aureus* undergoes normal cell division

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Abstract

DivIVA is involved in placement of the division septum and chromosome segregation in *Bacillus subtilis* and it plays important roles in cell division or morphogenesis in diverse Gram-positive bacteria. In *Staphylococcus aureus*, DivIVA is localized at the division septum, but it does not colocalize with the chromosomal origin of replication, as labeled with SpoOJ protein. Unexpectedly, a divIVA null mutant is not impaired in growth, nor is it affected in chromosome segregation or cell morphology.

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1. Introduction

When most bacteria divide, they usually generate two identical daughter cells. For that purpose the division plane, initiated by the polymerization of tubulin-like protein FtsZ, must be accurately placed in the middle of the parent cell. In *Bacillus subtilis*, regulation of the position of the Z ring is achieved by the combined effect of nucleoid occlusion and of the Min system [1,2]. MinC and MinD act together as a negative regulator of FtsZ ring assembly and are recruited to the cell poles by DivIVA. The presence of DivIVA-MinCD at the poles prevents future division at those sites [3–5]. More recently a second, non-essential function of DivIVA in chromosome segregation during *B. subtilis* sporulation, was identified: DivIVA seems to interact with the chromosome segregation machinery to help position the oriC region of the chromosome at the cell pole, in preparation for polar division [6–8]. Interestingly, some organisms, such as *Staphylococcus aureus*, contain divIVA, but do not contain minCD. The *S. aureus* DivIVA protein is 41 amino acids longer than the *B. subtilis* one but the degree of similarity of the sequence common to the two organisms is 72%. *S. aureus* divides in an unusual way, different from *B. subtilis*, as it switches division plane in three consecutive perpendicular orientations in successive division cycles [9,10]. Additionally, because staphylococcal cells are much smaller than those of *B. subtilis*, it seemed possible that chromosome segregation might require an extreme oriC separation system similar to the DivIVA-dependent one used during *B. subtilis* sporulation. We were therefore interested in determining the function of DivIVA in *S. aureus*, particularly to test whether it affects division plane selection or chromosome segregation.
2. Materials and methods

2.1. Construction of gfp fusions to divIVA and spoOJ

Plasmid pSG5082 was constructed cloning an erythromycin resistance marker, amplified from plasmid pMUTIN4 [11], into NaeI/SacI digested pSG1151 [12]. Plasmid pSG5082 was digested with Kpn I and Xho I and used to clone a complete copy of divIVA (amplified from chromosomal DNA of strain RN4220 using primers SADivIVAP3K – gtaggtacctgatcatactttccttggttgctcaatgc and SADivIVAP4Xh – tggactcagcttcagttgtctgtaac; Kpn I and Xho I restriction sites used for cloning are underlined in the primer sequence) upstream of gfp. This plasmid, pDivIVA-3, was then electroporated into S. aureus strain RN4220 (R. Novick) as previously described [13]. As pSG5082 does not replicate in S. aureus, the transformants have pDivIVA-3 integrated into the chromosome. The correct integration was confirmed by PCR.

Plasmid pSpoOJ-2 was constructed by amplifying a full copy of spoOJ from strain RN4220, using primers SASpoOJP3 – cgctgctcagttgtctgtaac and SASpoOJP2 – gcgctcgagcttcttagttgtttc, and cloning it into Xho I digested pSG5082. This plasmid was then electroporated into RN4220 resulting in strain RNPSpoOJ-2.

2.2. Fluorescence microscopy

Strains were grown to mid-exponential phase in tryptic soy broth (TSB, Difco) at 30 °C, before being observed by fluorescence microscopy using an Axiosvert 135TV inverted microscope (Zeiss) either on glass slides or on a thin film of 1% agarose in TSB prepared using Gene Frames (ABgene). Image acquisition was done using a Sony CoolSnap HQ cooled CCD camera (Roper Scientific) attached to the microscope and Metamorph (Universal Imaging). When necessary cells were stained with the DNA dye Hoechst 33342 (1 μg/ml; Molecular Probes) or Nile Red (5 μg/ml; Molecular Probes). The localization of DivIVA at the nascent septum was detected with anti-DivIVA antiserum raised against B. subtilis DivIVA protein.

2.3. Construction of a divIVA null mutant

Two fragments of 1.5 kb were ampliﬁed by PCR from chromosomal DNA of strain RN4220, corresponding to the upstream region (using primers gcgagatctcactgatcatacaagaaggttc and gcagctcagttgattctctcttcattacaa) and downstream region (using primers actgctgatctgatcatacaagaaggttc and gcagctcagttgattctctcttcattacaa) of divIVA. Restriction sites used for cloning are underlined in the primers sequence. These fragments were cloned into the non-replicative plasmid pORI280 [14] resulting in plasmid pDivIVA-15. This plasmid was electroporated into S. aureus and transformants were selected using erythromycin (10 μg/ml) in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 0.012%; Melford Laboratories). The correct insertion of pDivIVA-15 was confirmed by PCR, and one transformant was subsequently grown in the absence of antibiotic for 30–35 generations. Different dilutions of the final culture were plated in tryptic soy agar (TSA, Difco) containing X-gal (0.012%) and white colonies, which should have lost the plasmid, were streaked for further analysis. PCR using two pairs of primers, one pair internal (ataggtacctgtgtctgagaaggttc and tgactcagcttcagttgtctgtaac) and one pair external to the divIVA gene (cagcaaaagagcagtcacaac and tggactcagcttcagttgtctgtaac) was used to identify strains in which the gene was deleted from the chromosome.

Strains without divIVA were further analyzed by Western blotting. Total cell extracts, obtained after breaking the cells using glass beads in a FastPrep 120 cell disrupter (Qbiogene), were loaded onto 12% SDS–PAGE gel, transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences), and DivIVA was detected with anti-DivIVA antiserum raised against the B. subtilis protein.

3. Results and discussion

3.1. Localization of divIVA in S. aureus

To determine the localization of DivIVA, we introduced a plasmid encoding a C-terminal GFP fusion of the protein into the chromosome of S. aureus strain RN4220. The resulting strain, RNPSpoOJ-3, was used for fluorescence microscopy studies of DivIVA (Fig. 1(A)). DivIVA-GFP localized early to the division septum of S. aureus. When the nucleoid started to elongate in preparation for cell division, even before a complete septum could be seen by membrane staining, DivIVA appeared as two spots, which likely corresponded to a ring around the future division plane (for example cell labeled “a”, in Fig. 1(A)). A complete ring could be seen in cells in which the division plane was parallel to the microscope slide (for example cell labelled “b”, in Fig. 1(A)).

The localization of DivIVA at the nascent septum when the nucleoids elongate along an axis perpendicular to division plane, did not seem compatible with a possible role for DivIVA in localization of the origins of the chromosome. To clarify this issue, we constructed a strain containing a SpoOJ-GFP fusion protein – RNPSpoOJ-2. SpoOJ is a DNA binding protein that binds to the oriC region of the chromosome in B. subtilis and can therefore be used as a marker for the chromosomal origin of replication [15,16].
Observation of strain RNpSpoOJ-2 by fluorescence microscopy (Fig. 1(B)) showed that when the septum is complete (and therefore DivIVA is localized in a ring around the septum), the origins of replication are located near the “poles” of the cells, i.e., maximally distant from the septum. The fact that DivIVA does not localize near the origin of replication indicates that it is unlikely to have a similar function in chromosome segregation as in *B. subtilis* sporulation. However, as it seemed to localize early to the future division plane, it might have some role in the formation of the septum.

### 3.2. The divVA gene is not essential in *S. aureus*

To study the function of DivIVA in *S. aureus* we constructed a *divIVA* null mutant by removing the entire gene from the chromosome (see strategy in Fig. 2(A) and Methods): briefly, two DNA fragments corresponding to the upstream and downstream regions of *divIVA* were cloned into the non-replicative plasmid pORI280 [14] resulting in plasmid pDivIVA-15. As pORI280 contains the *lacZ* reporter gene, integration of the plasmid into the staphylococcal chromosome resulted in erythromycin resistant colonies that were blue. One transformant was subsequently grown in the absence of antibiotic for 30–35 generations to promote the loss of the plasmid. Colonies that had lost the plasmid (approximately 0.2%) no longer contained the *lacZ* gene and were therefore white. The ability to screen directly by color for the loss of the plasmid greatly facilitates the construction of null mutants in *S. aureus*, since excision of plasmids occurs at a very low frequency. Loss of the plasmid could give rise to either wild-type colonies or *divIVA* null mutants, and therefore PCR was used to identify strains in which the gene was deleted from the chromosome, which were 5 out of the 15 colonies analyzed. PCR with primers internal to *divIVA* did not give any product when chromosomal DNA from strain RNpDivIV-1-15 was used as template. When primers external to *divIVA* were used, the band amplified from RNpDivIV-1-15 chromosomal DNA was approximately 0.6 kb shorter than that of parental strain RN4220, as expected if no *divIVA* was present in RNpDivIV-1-15 (Fig. 2(B)). It is worth to mention that the null mutant is left with no plasmid DNA in the chromosome, namely no resistance marker and therefore any eventual change in the phenotype should be due only to the absence of DivIVA. The absence of DivIVA was further confirmed by Western blot (Fig. 2(C)).

Mutation of *divIVA* in *B. subtilis* causes misplacement of the septum during cell division, resulting in the formation of small, circular, anucleate minicells [17]. In *Streptomyces coelicolor* [18] or *Brevibacterium lactofermentum* [19] *divIVA* appears to be an essential gene. In *Streptococcus pneumoniae*, the *divIVA* homologue is not essential, but its inactivation results in a strain with a doubling time of almost twice that of the parental strain [20]. However, we have determined the
growth rate of strain RNpDivIVA-15 at 37 °C in TSB by following the optical density at 600 nm and found that it is the same as that of its parental strain RN4220 (doubling time of approximately 28 min in both cases), indicating that not only divIVA is not essential in S. aureus but that its inactivation does not impair growth.

3.3. The divIVA null mutant has normal chromosome morphology

To determine the effect of the divIVA null mutation on chromosome morphology, the RN4220 parental strain and the RNpDivIVA-15 mutant were grown to exponential phase in TSB, stained with the DNA dye Hoechst 33342 and the membrane dye Nile Red and observed by fluorescence microscopy (Fig. 3). The chromosomes had a normal appearance in the divIVA null mutant and we could not see an increase in the number of anucleate cells.

3.4. The divIVA null mutant has normal morphology when observed by electron microscopy

To determine the cell morphology of the divIVA null mutant, RN4220 and RNpDivIVA-15 cultures, grown to mid exponential phase were processed for electron microscopy. Extensive analysis did not reveal alterations to the normal cell morphology (not shown).

Our results indicate that when S. aureus is grown in rich medium, DivIVA does not seem to have an important role in chromosome segregation. Its localization in staphylococcus does not coincide with the location of the chromosome origins of replication and divIVA null mutant cells have normal chromosome morphology. The fact that DivIVA localizes to the division septum may suggest a role in septum formation, although the septa looked normal both by electron and fluorescence microscopy. DivIVA has been shown to be important in cell cycle processes in several diverse bacteria, and its absence is either lethal or severely impairs growth [5,17–20]. It seems, however, that in S. aureus this is not the case. Cell division has been mainly studied in the model organisms B. subtilis and Escherichia coli. Our results reinforce the importance of studying this fundamental process in other organisms, with different cell morphologies and modes of division. It seems of
particular importance to do these studies in pathogenic bacteria, such as *S. aureus*, for which conclusions regarding essential genes or fundamental cell processes are often used as indication of potential targets for the development of new antimicrobial therapies.

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**References**


