Characterization of divIVA and Other Genes Located in the Chromosomal Region Downstream of the dcw Cluster in Streptococcus pneumoniae†

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Received 16 June 2003/Accepted 14 July 2003

We analyzed the chromosome region of Streptococcus pneumoniae located downstream of the division and cell wall (dcw) cluster that contains the homolog of the Bacillus subtilis cell division gene divIVA and some genes of unknown function. Inactivation of divIVA in S. pneumoniae resulted in severe growth inhibition and defects in cell shape, nucleoid segregation, and cell division. Inactivation of the ylm genes resulted in some morphological and/or division abnormalities, depending on the inactivated gene. Transcriptional analysis revealed a relationship between these genes and the ftsA and ftsZ cell division genes, also indicating that the connection between the dcw cluster and the divIVA region is more extensive than just chromosomal position and gene organization.

Historically, most of the available information about bacterial cell division comes from the intensively studied gram-negative rod Escherichia coli and the gram-positive rod Bacillus subtilis (reviewed in references 1, 9, 10, 15, 17, and 24). This situation is changing, due to both the increasing interest in the field of cell division and the availability of genomic data. Most of the cell division genes have now been identified in a wide range of bacteria, and despite the differences observed among species, many of these genes are found organized in a chromosomal region corresponding to the 2-min region of the E. coli chromosome known as the dcw (for “division and cell wall”) cluster.

Notwithstanding the large amount of sequence information, very little is known about the molecular mechanism that regulates cell division in bacteria other than the model organisms and cell division in gram-positive cocci remains poorly understood. However, some extrapolations from the analysis of the Fts proteins that are essential for cell division, in particular the FtsZ protein, the major component of the septal ring structure (15), suggest that the basic mechanism involved in septum formation should not differ from cocci to rods.

A more intriguing problem and one that shows significantly less conservation is how different bacteria operate the division site selection mechanism that ensures correct FtsZ positioning. In E. coli, the correct division site at the center is selected as the functional counterpart of the missing MinE in B. subtilis (3, 7, 18, 19).

Recently, we identified a Streptococcus pneumoniae gene encoding the B. subtilis DivIVA homolog (20). We noted that divIVA is the last gene of a region located downstream of the cell division genes ftsA to ftsZ and is conserved in the same position in a number of gram-positive cocci. In addition to divIVA, the region contains some open reading frames of unknown function, designated ylmD, ylmE, ylmF, ylmG, and ylmH, all transcribed in the same direction as the dcw genes. In streptococci the first gene of the cluster, ylmD, is missing (20).

Both chromosomal position and gene organization suggested that these genes may be involved in cell division, sparking our interest in their characterization.

The function of divIVA and the ylm genes in S. pneumoniae was analyzed by insertion-deletion mutagenesis. Constructs for gene inactivation were obtained by a two-step PCR method. Three sets of primers (sequences available on request) were used for each construct. PCR products were amplified from the appropriate template DNA by using 1 U of Taq polymerase (Perkin-Elmer) in a Hybaid thermocycler. The first set of primers was used to amplify fragment 1, corresponding to the 5’ end of the gene to be inactivated; the second set was used to amplify the cat cassette from pR326 (4); and the third set was used to amplify fragment 2, corresponding to the 3’ end of the gene to be inactivated. PCR-derived fragments were purified by using a PCR purification kit (Qiagen), mixed in the molar ratio of 1:1:1, and reamplified in a second run with the external primers.

Linear constructs containing the cat cassette in the middle were obtained for the inactivation of ylmE, ylmF, ylmG, ylmH, and divIVA and used to transform S. pneumoniae wild-type Rx1 strain (20) as previously reported (23). Transformants selected on tryptic soy agar plates supplemented with 5% (vol/vol)
defibrinated sheep blood containing chloramphenicol (4 µg/ml) were obtained in all cases, indicating that none of these genes is essential to S. pneumoniae.

For each disruption, at least four transformants showing the correct insertion, as verified by PCR, were further analyzed. The different phenotypic characteristics of the divIVA::cat and each of the ylm::cat mutants relative to those of the wild-type Rx1 strain are discussed below and summarized in Table 1.

The divIVA::cat mutants, although viable, revealed the most dramatic defects with respect to growth, morphology, division, and nucleoid distribution. When cultivated in tryptic soy broth (TSB) medium, divIVA::cat mutants grew significantly slower then the wild type (Table 1). Phase-contrast microscopy and 4′,6′-diamidino-2-phenylindole (DAPI) staining showed that divIVA::cat mutants grew as chains of unseparated cells often devoid of nucleoid (Fig. 1). Electron microscopy confirmed the presence of chains with individual cells that lost the lancet-like shape characteristic of pneumococci and showed altered cell diameter, irregular margins, and unclosed division septa at midcell (Fig. 2).

Although the divIVA null phenotype may indicate a requirement of divIVA for proper septum formation and assembly in S. pneumoniae, as suggested by the presence of incomplete septa in cells lacking functional DivIVA, the possibility that both these defects and the presence of anucleate cells may originate from a defect in chromosomal segregation cannot be completely excluded. Additionally, the divIVA null phenotype in S. pneumoniae differs substantially from the divIVA null phenotype in B. subtilis (3, 7) and the previously proposed functional homology of DivIVA with MinE does not seem to apply to S. pneumoniae.

Recently, Thomaides et al. (25) have shown a second distinct function in chromosomal segregation for the B. subtilis DivIVA protein during sporulation. This second function of B. subtilis DivIVA better agrees with some features shown by S. pneumoniae divIVA null mutants, raising the possibility that the primary role of DivIVA is in chromosomal segregation. Consistent with this possibility is the presence of divIVA in bacteria, including gram-positive cocci, that seem to lack an equivalent of the min genes.

Previous protein sequence similarity searches showed that DivIVA has homologs among the gram-positive bacteria (8, 20) and possibly among some eukaryotic coil-coiled proteins (8) but not among gram-negative bacteria. Interestingly, when the DivIVA homologs from high-G+C-content gram-positive bacteria were used as a query sequence in BLAST searches, a significant degree of similarity (data not shown) with the E. coli TolA (12) was detected. In support of this observed similarity is the recently reported role of TolA in E. coli, in which mutations or deletions in domains II or III of the protein were shown to cause cell division defects (22) very similar to those of the divIVA null mutant phenotype reported here for S. pneumoniae. However, further studies to identify partner proteins that interact with DivIVA, suppressors of the divIVA null phenotype, and cellular localization of DivIVA in S. pneumoniae should help to verify the relationship between TolA and DivIVA and to clarify the precise role of DivIVA in cocci.

Inactivation of the ylm genes resulted in some morphological and/or division abnormalities, depending on the inactivated gene. Growth in TSB medium showed that all ylm mutants had doubling times similar to that of the wild type, with the exception of ylmG::cat mutants (Table 1). However, phase-contrast microscopy, DAPI staining of the nucleoids, and electron microscopy showed a distinct phenotype for each ylm mutant analyzed by phase-contrast microscopy, DAPI staining of the nucleoids, and electron microscopy. This second function of DivIVA better agrees with some features shown by S. pneumoniae divIVA null mutants, raising the possibility that the primary role of DivIVA is in chromosomal segregation. Consistent with this possibility is the presence of divIVA in bacteria, including gram-positive cocci, that seem to lack an equivalent of the min genes.

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S. pneumoniae ylmE::cat mutant cells were similar in shape to but, on average, slightly larger than wild-type cells. However, the fluorescence of their nucleoids appeared greatly reduced (Fig. 1). Electron microscopy of ylmE::cat cells confirmed the larger size of this mutant and the presence of internal zones that appeared to be less electron dense (Fig. 2).

S. pneumoniae ylmF::cat mutants analyzed by phase-contrast microscopy were morphologically different, showing the presence of some sausage-like cells and occasionally of elongated cells and minicells. DAPI staining revealed normal fluorescence in all cells, including minicells, where a guillotine effect

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**TABLE 1. Relevant features of the null mutant phenotypes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (min)</th>
<th>Phase-contrast morphology</th>
<th>Nucleoid fluorescence</th>
<th>Shape</th>
<th>Cell diameter (µm)</th>
<th>Septum</th>
<th>Transformability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rx1</td>
<td>32</td>
<td>Diplococci</td>
<td>Parental strain</td>
<td>Normal</td>
<td>0.54 ± 0.022</td>
<td>Symmetrical and well defined</td>
<td>+</td>
</tr>
<tr>
<td>Rx1 ylmE::cat</td>
<td>36</td>
<td>Diplococci</td>
<td>Significantly reduced</td>
<td>Normal</td>
<td>0.60 ± 0.020</td>
<td>Thinner</td>
<td>+</td>
</tr>
<tr>
<td>Rx1 ylmF::cat</td>
<td>36</td>
<td>Chains</td>
<td>As parental</td>
<td>Altered</td>
<td>0.64 ± 0.017</td>
<td>Thinner and incomplete</td>
<td>–</td>
</tr>
<tr>
<td>Rx1 ylmG::cat</td>
<td>42</td>
<td>Diplococci and tetrads</td>
<td>As parental</td>
<td>Normal</td>
<td>0.55 ± 0.013</td>
<td>Thinner</td>
<td>+</td>
</tr>
<tr>
<td>Rx1 ylmH::cat</td>
<td>32</td>
<td>Diplococci and chains</td>
<td>As parental</td>
<td>Normal</td>
<td>0.56 ± 0.014</td>
<td>Thinner</td>
<td>+</td>
</tr>
<tr>
<td>Rx1 divIVA::cat</td>
<td>56</td>
<td>Chains</td>
<td>Absent in some cells</td>
<td>Altered</td>
<td>0.62 ± 0.022</td>
<td>Incomplete and occasionally oblique</td>
<td>+</td>
</tr>
</tbody>
</table>

* * DAPI stain fluorescence was used to determine the presence and location of the nucleoid.
* b Cell diameters are given as the means ± the standard deviations calculated by measuring S. pneumoniae cells at the same stage of division.
* c +, colonies were recovered on plates after transformation with plasmid or linear DNA; –, no colonies were recovered on plates after transformation with plasmid or linear DNA.

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FIG. 1. Phase-contrast analysis of the Rx1 wild-type strain and the insertion-deletion mutants. Bacterial cells were cultured in TSB medium, sampled at selected times during the exponential phase of growth, fixed with 1% (vol/vol) formaldehyde for 15 min at room temperature, treated with DAPI as previously described (2), and examined by using an Axioskop HBO50 equipped with a Plan-Neofluar 100× oil lens. For each strain, phase-contrast (left panels) and DAPI stain fluorescence (right panels) micrographs are shown. The scale bars correspond to 3 µm.
FIG. 2. Electronic transmission analysis of the Rx1 wild-type strain and of the insertion-deletion mutants. Cells were cultured in TSB medium to mid-exponential phase, fixed in 1% (vol/vol) glutaraldehyde for 2 h at room temperature, processed as previously described (11), and then observed and photographed in a Zeiss EM 10 electron microscope. The abnormalities of various null mutants are described in the text and summarized in Table 1. The scale bars correspond to 0.3 μm.
on the nucleoid was observed (Fig. 1). In the electron micro-
graph of ylmF::cat mutants, the multiseptate sausage-like ele-
ments with multiple and thinner septa at an early stage of
constriction were evident (Fig. 2).

S. pneumoniae ylmG::cat mutants did not show any substantial
difference in morphology or fluorescence. However, packs of
cells resembling tetrads were seen in addition to diplococci
in phase-contrast and fluorescence micrographs (Fig. 1). Thin-
ner septa were observed in the electron micrographs (Fig. 2)

S. pneumoniae ylmH::cat mutants grew as diplococci or short
chains of cells showing the typical pneumococcal shape and
normal fluorescence (Fig. 1). However, ylmH null cells started
lysing before the end of the exponential phase, earlier than the
physiological lysis of the wild type. Electron microscopy re-
vealed the presence of diplococci where the most appreciable
difference was the presence of thinner septa (Fig. 2).

In addition to the single inactivations, double knockouts
were constructed for each individual ylm::cat mutant. A divIVA
fragment containing an erm cassette from plasmid pVA838
(16) was used to transform each ylm::cat mutant. Double null
mutants were selected on tryptic soy agar blood plates contain-
ing chloramphenicol (4 µg/ml) and erythromycin (0.2 µg/ml)
for all recipient single null mutants with the exception of the
ylmF::cat strain (Table 1).

All the double mutants obtained (ylmE::cat-divIVA::erm, ylmG::cat-divIVA::erm, and ylmH::cat-divIVA::erm) showed a
divIVA null phenotype, indicating the epistatic effect of divIVA
with respect to the other genes and supporting the possibility
that the single gene inactivations lack a polar effect on the
downstream region.

In the absence of obvious cell division mutants in S. pneu-
moniae and in other gram-positive cocci, the ylm null pheno-
types were less evident than the divIVA null phenotype and the
results were more difficult to interpret. However, as suggested
by the ylmF::cat phenotype, ylmF may be involved in determin-
ing the frequency and position of the septum, a possibility in
support of the observation that other, still unknown factors are
involved in this process (14). Moreover, although the pheno-
type of the single ylmE and ylmG null mutants did not give us
any conclusive evidence, the disruption of the pairs ylmE::erm
and ylmG::cat showed that double mutants divide in multiple
planes, in contrast to the single plane used by streptococci (not
shown).

The tight organization of the genes located downstream of
the dcv cluster suggested that in S. pneumoniae these genes are
transcribed together and possibly also with the adjacent cell
division genes ftsA and ftsZ.

To investigate this possibility, S. pneumoniae cells were
grown in TSB medium to the mid-exponential phase and total
RNA was extracted and used in reverse transcription (RT-
PCR with the cMaster RT<sub>prim</sub>PCR system. RT-PCR products
of the expected sizes were amplified in all cases (data not
shown), indicating a transcriptional relationship between these
genes. Therefore, to determine if the insertional mutation in
the upstream gene would abolish the transcription of the
downstream genes by a polar effect, we analyzed the expression
of ylmF, ylmG, ylmH, and divIVA genes in the wild-type Rx1
strain and in each of the insertion-deletion mutants by RT-
PCR. As shown in Fig. 3, we found that, without exception, all
the downstream genes were still expressed in each of the mu-
tants, indicating that downstream transcription was not abol-
ished by the cat cassette insertion.
Taken together, our results indicate that the relationship between the dcw cluster and the divIVA region is more extensive than just chromosomal position and gene organization and suggest a complex pattern of transcriptional regulation between the last genes of the dcw cluster and the genes located immediately downstream.

This work represents an initial step in elucidating cell division in gram-positive cocci. In particular, it underlines the fact that the molecular mechanisms at the base of this process cannot be extrapolated solely from the information available from other bacteria, including *B. subtilis*. Thus, *S. pneumoniae* can provide a useful model among the gram-positive cocci, due to both the ease of genetic manipulation and the simple mode of division.

This work was supported by Cell Factory Contract no. CT96-0122 from the European Commission (DGXII) Biotechnology Program and by 60% MURST funds to O. Massidda.

We gratefully acknowledge C. Zancanaro and F. Merigo of the Dipartimento di Scienze Morfologico-Biomediche, Università di Verona, and A. Riva of the Servizio di Microscopia Elettronica, Dipartimento di Citomorfologia, Università di Cagliari, for their support in electron microscopy. We thank P. E. Varaldo for critical review of the manuscript.

REFERENCES